THE ROLE OF METAL IONS IN THE ACTIVATION OF CREATINE KINASE

A kinetic study of the magnesium activation of the reaction catalysed by ATP:creatine phosphotransferase (creatine kinase).

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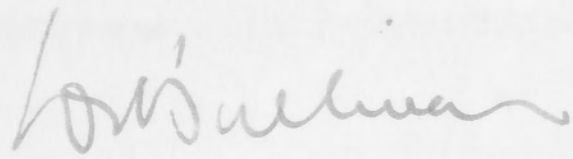
This thesis embodies the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from February, 1960, to February, 1963, during the tenure of an Australian National University Research Scholarship in 1960 and 1963 and a General Motors-Holden's Postgraduate Research Fellowship for 1961 and 1962.

STATEMENT

The regulations of the Australian National University require that a statement be made describing which parts of the work described in this thesis have been carried out by myself. This may best be done by quoting from a letter from my supervisor, Dr. J. F. Morrison, to the Registrar of the University:

"..... The results reported in Chapters IV, V and VI were obtained in collaboration with me. The candidate's contribution to the early work (Chapter IV) was that of a junior worker, whilst his contribution towards the remainder was that of a co-worker. The work described in Chapters II, III and VII was carried out independently by the candidate ....".

Candidate's signature:



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I should like to express my appreciation of the guidance and encouragement shown to me by my supervisor, Dr. J.F. Morrison, during this work.

I am indebted to a number of people for assistance at various stages of the work and acknowledgement, where appropriate, has been made in the text of the thesis. In particular, I should like to thank Dr. D.D. Perrin for his supervision of the stability constant measurements described in Chapter II, Professor A.G. Ogston for many discussions on enzyme kinetics and Professor A.H. Ennor for his general interest in the project.

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PREFACE

The enzyme nomenclature in this thesis is in accordance with the recommendations of the Report of the Commission on Enzymes of the International Union of Biochemistry (I.U.B. Symposium, 1961). At first mention the full name, trivial name and number of the enzyme are given; otherwise the trivial name has been used.

Temperatures are expressed in °C.

Figures and tables are presented on separate pages, a particular figure or table following immediately the page on which first reference to it has been made.

The following abbreviations are used:

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
EDTA	ethylenediaminetetraacetic acid (sodium salt)
NAD	nicotinamide-adenine dinucleotide
PC	phosphorylcreatine
TRIS	tris(hydroxymethyl)aminomethane

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GENERAL INTRODUCTION

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GENERAL INTRODUCTION

Preamble

With the preparation of purified enzymes it has become apparent that in a number of cases some dialysable substance(s), essential to the activity of the enzyme, may be lost, either partially or wholly, during the purification procedure. Thus, the activity of the purified enzyme may be either restored or increased by the addition of low molecular weight compounds.

Divalent metal ions form an important group of such cofactors or coenzymes and are required, in particular, for those enzymic reactions which involve the transfer of a phosphoryl group. This requirement is usually met by Mg^{2+} and often by Mn^{2+}. Ca^{2+} ions are active with some phosphoryl transferring enzymes and, in certain instances, other divalent ions such as Fe^{2+}, Zn^{2+} and Co^{2+} may function as activators (Bock, 1960).

The role of metal ions in enzymic catalysis is not well understood. This is particularly true where the metal ion does not complex strongly with the enzyme,

as with those enzymes concerned with the transfer of a phosphoryl group, and it is not possible to deal with a "metalloenzyme" entity (cf. Vallee, 1955).

The work presented in this thesis is concerned with a kinetic study of the Mg^{2+} activation of the enzyme ATP:creatine phosphotransferase (creatine kinase; 2.7.3.2). This enzyme, which catalyses the reversible transfer of a phosphoryl group, according to the equation

$$\text{ATP} + \text{creatine} \underset{\text{reverse}}{\overset{\text{forward}}{\rightleftharpoons}} \text{ADP} + \text{phosphorylcreatine}$$

is similar to other enzymes carrying out reactions of this type, in that it has an absolute requirement for a divalent metal ion. This requirement can be met by Mg^{2+}, Mn^{2+}, Ca^{2+} or Co^{2+}.

The work carried out can be divided into two sections, namely, the determination of the stability constants of the complexes $MgADP^-$ and $MgATP^{2-}$ (Chapter II) and the kinetic studies on creatine kinase (Chapters III to VII). The principal object of the stability constant work was to obtain values for use in the kinetic experiments, though the work has been expanded beyond this requirement. The kinetic approach was first applied to the reverse reaction catalysed by creatine kinase. The results obtained were compatible with the

interaction of the species, free Mg^{2+}, free ADP^{3-} and the $MgADP^-$ complex, with the enzyme. This work is described in Chapter IV. In Chapter III some observations on the residual activity of the enzyme are reported. Chapter V describes experiments designed to elucidate the nature of the inhibition by excess Mg^{2+} and excess ADP, with respect to both $MgADP^-$ and phosphorylcreatine (PC). Because the results obtained cast some doubt on the interpretation of the results in Chapter IV, a reassessment of the kinetics of the reverse direction was carried out as described in Chapter VI. Chapter VII describes results obtained from studies of the forward direction and in Chapter VIII the conclusions drawn from the thesis are discussed in relation to other work carried out on creatine kinase.

ENZYMIC PHOSPHORYL TRANSFER REACTIONS

The majority of enzymic phosphoryl transfer reactions are concerned with the transfer of the terminal phosphoryl group of a nucleoside-triphosphate to an acceptor molecule to yield the phosphorylated derivative of the acceptor molecule. Such reactions may be represented by the general equation

$$NTP + A \rightleftharpoons NDP + A\text{-}P$$

Experimental evidence for the idea that the transfer occurs as a result of nucleophilic attack on the P atom

of the terminal phosphoryl group, has come largely from O^{18} studies on the reactions catalysed by such enzymes as ATP:D-hexose 6-phosphotransferase (hexokinase; 2.7.1.1), ATP:AMP phosphotransferase (myokinase; 2.7.4.3), ATP:pyruvate phosphotransferase (pyruvate kinase; 2.7.1.40), ATP:D-3-phosphoglycerate 1-phosphotransferase (3-phosphoglycerate kinase; 2.7.2.3) and creatine kinase (Cohn, 1959; Harrison, Boyer and Falcone, 1955). Thus, reactions carried out in the presence of H_2O^{18} showed that there was a direct transfer of the terminal phosphoryl group of ATP to the acceptor molecule, by virtue of the bond cleavage occurring at O-P. No exchange between O^{18} and either the oxygen atoms of the phosphoryl group or of the acceptor molecule was observed.

The transfer of a phosphoryl group has been postulated as occurring either via a double displacement mechanism involving the formation of a phosphoenzyme intermediate or by a single displacement mechanism, whereby a direct transfer of the phosphoryl group from donor to acceptor occurs. This latter mechanism appears to be operative for the reactions catalysed by creatine kinase and pyruvate kinase. Thus, Noda, Nihei and Morales (1960) failed to demonstrate exchange between (β-^{32}P)ADP and ATP in the presence of creatine kinase. This confirmed the earlier product inhibition studies

(Kuby, Noda and Lardy, 1954b) which had indicated that ADP and ATP competed for a common binding site on the enzyme surface, while an independent site was concerned with the binding of creatine and phosphorylcreatine (PC). The two sites were considered to be in juxtaposition in relation to the enzyme and to have a common area for both the terminal phosphate group of ATP and the phosphoryl group of PC. Thus, phosphoryl group transfer could occur as a single displacement mechanism.

Harrison *et al.* (1955) did not obtain any evidence for the formation of a phosphoenzyme intermediate in the reaction catalysed by pyruvate kinase, the enzyme being unable to catalyse an exchange of pyruvate-C^{14} with phosphoenolpyruvate. The product inhibition studies of Reynard, Hass, Jacobsen and Boyer (1961) indicated a single displacement mechanism as with creatine kinase. Thus, the inhibition by ATP was competitive with respect to both ADP and phosphoenolpyruvate. This finding led the authors to conclude that the transferable phosphoryl group of the latter occupied the same space as the terminal phosphoryl group of ATP. The kinetic studies were substantiated by direct substrate binding measurements which showed that pyruvate and ADP combined independently with the enzyme and that ATP decreased the binding of both

phosphoenolpyruvate and ADP.

By contrast, it has been postulated that a number of enzymic phosphoryl transfer reactions, in which ATP is not involved, occur via a phosphoenzyme intermediate. Evidence for this in the case of muscle D-glucose-1,6-diphosphate:D-glucose-1-phosphate phosphotransferase (phosphoglucomutase; 2.7.5.1) has been obtained from kinetic and equilibrium studies (Najjar and Pullman, 1954; Sidbury and Najjar, 1957) and from the observation that exchange occurred between ^{32}P labelled glucose-1-phosphate and the enzyme (Jagannathan and Luck, 1949). The phosphoryl group appears to be linked to the protein through a serine residue as acid hydrolysis (Anderson and Jolles, 1957) or proteolysis (Kennedy and Koshland, 1957) yielded O-phosphoserine or peptides containing O-phosphoserine. This has been confirmed by Milstein and Sanger (1961), who mapped the active centre of phosphoglucomutase, obtaining the sequence

-Thr-Ala-Ser.P-His-Asp .

Evidence for the participation of a phosphoenzyme in enzymic phosphoryl transfer reactions based principally on the isolation of ^{32}P enzymes obtained after incubation with the ^{32}P labelled substrates, has been obtained for a number of other enzymes, including

yeast phosphoglucomutase (McCoy and Najjar, 1959), orthophosphoric monoester phosphohydrolase (alkaline phosphatase; 3.1.3.1) (Agren, Zetterqvist and Ojamäe, 1959), D-2,3-diphosphoglycerate:D-2-phosphoglycerate (phosphoglyceromutase; 2.7.5.3) (Pizer, 1960), phosphoserine phosphohydrolase (phosphoserine phosphatase; 3.1.3.3) (Neuhaus and Byrne, 1959) and D-glucose-6-phosphate phosphohydrolase (glucose-6-phosphatase; 3.1.3.9) (Hass and Byrne, 1960).

Before discussing the role of metal ions in phosphoryl transfer reactions, it is useful to consider the activation of enzymes by metal ions from a general viewpoint.

General aspects of activation by divalent metal ions.

The absolute requirement of an enzyme for a divalent metal ion is becoming an increasingly familiar phenomenon. For instance, Dixon and Webb (1958a) have listed nearly 100 enzymes, not including the haem-type enzymes, which either require the addition of divalent metal ion for activation or contain an intrinsically bound metal ion.

It should be pointed out that the requirement of an enzyme for the addition of a metal ion should be confirmed by studies on a pure enzyme, as otherwise the observation of an apparent activating effect cannot be

taken as conclusive evidence for the participation of the metal in the catalytic mechanism. Thus, an apparent activation could be due to a number of indirect effects, such as the removal of inhibitor compounds as their metal complexes, the displacement of inhibitor metals combined with the enzyme or the substrate, or the stabilisation of the enzyme (Malmström and Rosenberg, 1959).

It is clear that, if it has been established that the metal ion is an essential cofactor for the enzymic reaction, then the metal ion must be, in some way, modifying one or more of the substrate, the enzyme or an enzyme-substrate complex. To discuss the possible effects of the metal ion, with reference to particular enzymes, it is convenient to differentiate between "metalloenzymes" and "metal-enzyme" complexes. This is a purely operational classification, based principally on the strength of binding of the metal to the enzyme, but has proved useful for comparative purposes (Vallee, 1955).

Metalloenzymes.

These are characterised by (1) firm attachment of the metal to the enzyme, presumably by a predominantly covalent bond, and (2) a stoichiometric relationship between the metal and the enzyme, so that the ratio of

[M] to [Enzyme] is a small integral number. Furthermore, the metal and the enzyme form a single entity in the natural state.

Though iron, copper and possibly molybdenum have been demonstrated to form metalloenzymes (Vallee, 1955; 1960; McElroy and Glass, 1950), the most closely studied are yeast and liver alcohol: NAD oxidoreductases (alcohol dehydrogenase; 1.1.1.1), carboxypeptidase (3.4.2.1) and carbonate hydro-lyase (carbonic anhydrase; 4.2.1.1), all of which contain zinc. These zinc enzymes have been studied, with particular reference to the function of the metal ion, by Vallee's group at Harvard and by Lindskog and Malmström.

Analyses of the metal content have resulted in the establishment of the following relationships between metal and enzyme:

(CPD)Zn for carboxypeptidase (Vallee and Neurath, 1954; Vallee, 1955),

(YADH)Zn_4 for yeast alcohol dehydrogenase (Vallee and Hoch, 1955),

(LADH)Zn_2 for liver alcohol dehydrogenase (Vallee and Hoch, 1957),

(CA)Zn for carbonic anhydrase (Lindskog, 1960; Lindskog and Malmström, 1962).

That the metal is intimately concerned with the activity

of these enzymes has been strikingly illustrated with carboxypeptidase (Vallee, Rupley, Coombs and Neurath, 1960), where it was found that removal of the metal resulted in a concomitant loss of activity. The activity could be restored not only by the addition of Zn^{2+} but also by the addition of other metals of the first transition series; viz., Mn^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}, Cr^{3+}. Similar observations have been made for carbonic anhydrase (Lindskog and Malmström, 1962), the activity of the enzyme being shown to be a direct function of the metal content. In this case only Co^{2+}, besides Zn^{2+}, was found to restore the activity of the enzyme.

Unlike the above enzymes, removal of zinc from the alcohol dehydrogenases gives rise to irreversible inactivation, probably because the zinc is required for the maintenance of the structural integrity of the enzyme. This is certainly the situation for yeast alcohol dehydrogenase, where complete removal of the zinc, by prolonged dialysis against 1,10-phenanthroline, results in the irreversible dissociation of an enzyme of M.W. 150,000 to four inactive protein units of M.W. 36,000 (Kägi and Vallee, 1960).

Hoch and Vallee (1956) and Hoch, Williams and Vallee (1958) have presented kinetic evidence that Zn^{2+} assists in the binding of nicotinamide-adenine

dinucleotide (NAD) to yeast alcohol dehydrogenase. It was found that 1,10-phenanthroline caused an immediate inhibition of the enzyme, this inhibition being competitive with respect to NAD or $NADH_2$. Spectroscopic evidence for the formation of an enzyme-Zn-1,10-phenanthroline complex, implying the possible formation of an enzyme-Zn-NAD complex, has been obtained for both the yeast and the liver enzymes (Vallee, Coombs and Williams, 1958; Vallee and Coombs, 1959).

Evidence for other NAD dependent dehydrogenases being zinc metalloenzymes has also been obtained (Vallee, Hoch, Adelstein and Wacker, 1956) but detailed studies have not been carried out.

Metal-enzyme complexes.

For these enzymes, where the metal ion does not form a strong complex with the enzyme, chemical stoichiometry is difficult to establish. It has proved difficult to carry out direct studies on a metal-enzyme complex and most explanations for the function of the metal ion have been reliant on a study of the enzymic activity in the presence of added metal ion.

Where it has been shown that a metal ion is an essential cofactor for an enzymic reaction, it has become generally accepted that such a reaction proceeds via an active complex involving enzyme, metal ion and

substrate often referred to as an EMS complex. Because maximum activity of the enzyme requires relatively high concentrations of metal ion, the metal is available for reaction with the substrate, independent to the enzymic reaction. This has led to two principal points of view as to the function of the metal ion. Firstly, that the metal ion interacts with the enzyme to give an "active" enzyme. This could be due to a structural rearrangement, analogous to the role played by zinc in yeast alcohol dehydrogenase (Kägi and Vallee, 1960), or the metal may modify the enzyme so that the substrate can approach the active site. Alternatively, it has been postulated that a metal-substrate complex may form the "true" substrate of an enzymic reaction, i.e., the substrate may be modified both electronically and structurally, facilitating enzymic attack. It should be pointed out that the second possibility does not necessarily preclude interaction between the metal and the enzyme. These two proposals will be discussed in more detail in the next section with particular reference to the phosphoryl transfer enzymes.

The fact that the metal is capable of binding with both the substrate and the enzyme has led to the idea that the metal may form a preferential link between the two. The formation of such a "bridge"

structure was proposed by Smith (1951), from his work on the peptidases. There is little doubt that metal ions can assist in the binding of organic molecules to proteins (Klotz and Ming, 1954; Gurd and Wilcox, 1956) but the participation of bridge structures in enzymic catalysis has proved difficult to establish unequivocally.

Examples of enzymes requiring the presence of metal ions for activity are D-2-phosphoglycerate hydro-lyase (phosphopyruvate hydratase or enolase; 4.2.1.11), the peptidases and the phosphoryl transfer enzymes. Of these, phosphopyruvate hydratase has been studied in terms of its metal ion requirement by Wold and Ballou (1957) and by Malmström and his colleagues. It was found that the enzyme is activated by the divalent metal ions Mg^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Cd^{2+}, Co^{2+} and Ni^{2+} (Malmström, 1955a; Wold and Ballou, 1957). Evidence for interaction between the metal and the enzyme was obtained from kinetic studies with Zn^{2+} (Malmström, 1953, 1954) and substantiated by equilibrium dialysis and electron spin resonance measurements with Mn^{2+} (Malmström, Vänngard and Larsson, 1958). It was concluded that the active enzyme was a metal complex containing one mole of activating ion per mole of protein.

Experiments designed to test for the participation of a bridge structure in the reaction catalysed by

phosphopyruvate hydratase were carried out by Malmström (1955b). It was found that Zn^{2+} certainly facilitated the binding of the substrate, 2-phosphoglycerate, but that four molecules of substrate were bound to the enzyme even though the earlier studies had indicated that it had only one active site. Further, it was found that Zn^{2+} had a similar mediating effect on the binding of 2-phosphoglycerate to albumin. Thus, while the metal certainly appeared capable of forming a preferential link between the protein and the substrate, the evidence for the participation of such a complex in the catalytic reaction was not conclusive.

For paramagnetic ions, magnetic resonance techniques can give much information. With Mn^{2+} as the activating ion, Larsson-Raznikiewicz and Malmström (1961) concluded that 3-phosphoglycerate kinase reacted with $MnATP^{2-}$ and not with the free metal. Cohn and Leigh (1962), using nuclear magnetic resonance, arrived at a similar conclusion for creatine kinase with Mn^{2+}, though with phosphopyruvate hydratase the metal appeared to be reacting with the enzyme. The results with creatine kinase shall be discussed in more detail in the Conclusion.

All enzymic phosphoryl transfer reactions involving

the adenine nucleotides appear to show an absolute requirement for a divalent metal ion (Lardy, 1951). Because of the weak interaction between the metal and the enzyme, postulates concerning the function of the metal ion have been largely reliant on evidence from kinetic experiments.

<u>The mechanism of the metal activation of phosphoryl transfer enzymes: a comparison of the conclusion that the metal-nucleotide complex is the "true" substrate with the concept of the formation of an active metal-enzyme complex</u>.

Most of the early kinetic studies of enzymic phosphoryl group transfer reactions were carried out in the forward direction only, as ATP was available in a purified state some time before ADP. The observation by a number of workers that optimal activity was obtained when the concentration of ATP approximately equalled that of the added metal ion led to the hypothesis that the enzyme reacted with a metal-ATP complex. It was subsequently found that this relationship does not hold with ADP as the substrate. This latter finding has given support to the alternative postulate, that the function of the metal ion was to form an active metal-enzyme complex.

On the basis that the optimum Mg^{2+} concentration

for the reaction catalysed by ATP:D-fructose 1-phosphotransferase (ketohexokinase, fructokinase; 3.7.1.3) was dependent on the ATP concentration, and having demonstrated that Mg^{2+} formed a complex with ATP, Hers (1952) concluded that this complex was the true substrate for the reaction. Similar conclusions for hexokinase were reached by Liebecq (1953), who demonstrated maximum activity for this enzyme when the Mg:ATP ratio equalled unity. This concept has been extended to other phosphoryl transfer enzymes, e.g., ATP:D-gluconate 6-phosphotransferase (gluconokinase; 2.7.1.12) (Leder, 1957), Mg-activated ATP phosphohydrolase (Mg-activated actomyosin ATPase; 3.6.1.4) (Perry and Grey, 1950).

Kuby, Noda and Lardy (1954b) carried out kinetic investigations of a crystalline preparation of creatine kinase, studying the reaction in both directions. With ATP as the substrate, maximum activity occurred when the total concentration of Mg was equal to that of the total ATP, regardless of the concentration of the latter. This observation, together with the demonstration that the observed K_m value for ATP in the presence of a fixed total concentration of Mg was the same as that obtained for Mg in the presence of the same fixed concentration of ATP, led to the conclusion that $MgATP^{2-}$ was the true substrate of the reaction. Employment of total Mg

concentrations in excess of the optimum, resulted in a depression of the reaction velocity and this was attributed to the binding of a second molecule of Mg^{2+} by $MgATP^{2-}$ to form a "less active" Mg_2ATP complex. The experimental results for the reverse reaction were not so clear-cut though similar conclusions were reached. In this case maximum activity was reached with the Mg:ADP ratio somewhat greater than unity and the agreement of the respective K_m's was not as good. All results were referred to total concentrations of Mg and nucleotide and no attempt was made to differentiate between the different forms of the nucleotide in solution.

It should be pointed out that, at the time these results were obtained, the available estimates for the stability constants of the $MgATP^{2-}$ and $MgADP^{-}$ complexes, viz., approximately 850 and 400 M^{-1}, respectively (Burton and Krebs, 1953), were too low by some orders of magnitude (Burton, 1959; Bock, 1960; this thesis, Chapter II). On the basis of the available values, insufficient amounts of the nucleotides would have been present as the metal complexes to account for the similarity in the K_m values for total nucleotide and total metal. This was recognised by Noda, Kuby and Lardy (1954), who concluded that considerably higher values of the stability constants were necessary to fit

their results on the equilibrium of the creatine kinase reaction and that a re-evaluation might be necessary.

Studies on the kinetics of the forward reaction have been extended by Noda, Nihei and Morales (1960). It was found that a value of 90,000 M^{-1} had to be assigned to the stability constant of $MgATP^{2-}$ to fit the kinetic data. On this basis, good agreement between experimental and calculated velocities was obtained except when the total ATP concentration exceeded the total Mg concentration. The inhibition observed under these circumstances was attributed to the increased concentration of ATP^{4-}, which was considered to act as a weak inhibitor. No inhibitory effects of excess total Mg over total ATP were reported. For the reverse reaction (Nihei, Noda and Morales, 1961) it was concluded that $MgADP^{-}$ was the true substrate, with ADP^{3-} a competitive inhibitor, a value of 2,000 M^{-1} being assigned to the stability constant of $MgADP^{-}$.

Kinetic studies on ATP:arginine phosphotransferase (arginine kinase; 2.7.3.3) were carried out by Griffiths, Morrison and Ennor (1957), with particular reference to the activation by Mg^{2+}. In agreement with the results for creatine kinase, it was found that maximum activity was obtained for the forward reaction when the Mg:ATP ratio was unity. However, for the reverse reaction,

the ratio of Mg to ADP for maximum velocity varied from 3 to 8. These workers used the values of Burton and Krebs (1953) for the stability constants of $MgATP^{2-}$ and $MgADP^{-}$ to calculate the amount of free Mg^{2+} present. It was found that maximum velocity was obtained at the same concentration of free Mg^{2+} for both the forward and reverse directions. On this basis it was concluded that the function of Mg^{2+} was to form an active metal-enzyme complex, which then reacted with the free nucleotide. The interaction between Mg^{2+} and the two nucleotides was considered to be a side reaction. As mentioned above, the values used for the stability constants were much too low and thus the basis for the conclusions drawn by these workers is almost certainly incorrect.

The proposal that the metal ion forms an active metal-enzyme complex has received support from Geske, Ulbrecht and Weber (1957) and Nanninga (1959). The latter worker considered ATP^{4-} and $CaATP^{2-}$ as alternative substrates for the reaction catalysed by ATP phospho-hydrolase (Ca-activated myosin ATPase; 3.6.1.3), carrying out determinations of the stability constant of $CaATP^{2-}$ under the same experimental conditions as for the enzymic experiments. The conclusion was reached that the kinetic data could be explained from two points of view, viz., either an active $CaATP^{2-}$ complex reacts with the enzyme

or the free ATP^{4-} reacts with an active Ca-myosin complex. However, it was believed that the inhibitory effect of excess Ca^{2+} was explained more satisfactorily on the latter hypothesis. If the first mechanism was correct, it would have to be assumed that Ca_2ATP was formed and that this was inactive; in the case of the second mechanism, it would be expected that, with excess Ca^{2+}, less substrate would be available since all the ATP would exist as $CaATP^{2-}$. On this basis, a plot of activity against total calcium concentration gave good agreement between theory and the experimental results, particularly in predicting the position of the Ca-optimum (Nanninga, 1959; Fig. 8).

The interaction between the reaction components of creatine kinase.

At the time this work was commenced the two approaches outlined above had been considered to be virtually exclusive, so that only one form of the substrate was thought to be capable of reacting with the enzyme. More recently, Noda et al. (1960) and Nihei et al. (1961) have found it necessary to consider more than one species of the nucleotide. It is useful to illustrate the inherent weakness in the assumption that only one species reacts with the enzyme.

Consider the reaction catalysed by creatine kinase:

$$ATP + creatine \xrightleftharpoons{Mg^{2+}} ADP + phosphorylcreatine.$$

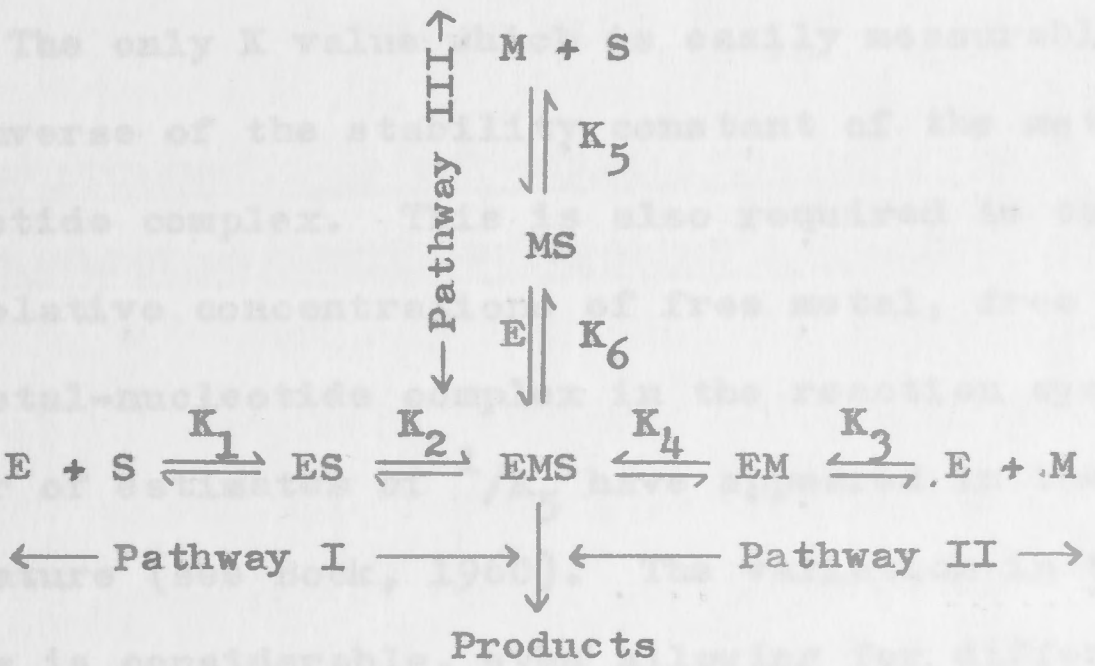
If the reaction is considered as taking place at pH 8.0, in a non-interacting buffer medium, then in the absence of metal ion the nucleotide would be present principally as ATP^{4-} with some $HATP^{3-}$, as the terminal phosphate group of ATP has a pK_a at approximately pH 7. On the addition of the metal ion, e.g., Mg^{2+}, the complexes $MgHATP^{-}$ and $MgATP^{2-}$ would be formed. The concentration of the various species would depend on the molar ratio of Mg^{2+} and ATP, $MgATP^{2-}$ being the predominant form of the nucleotide when Mg^{2+} was equal to or greater than the total ATP concentration. Thus, any kinetic study should at least consider the species, $MgATP^{2-}$ and the free forms, Mg^{2+} and ATP^{4-}. Further, the concentrations of these species should be known under the experimental conditions of the enzyme experiments.

Many kinetic studies (e.g., Reynard et al., 1961; Nihei, Noda and Morales, 1961; Fromm and Zewe, 1962) have been carried out with excess of divalent metal ion so that virtually all the nucleotide has been present as the metal complex, so that there was no need to consider any possible interaction of the free nucleotide with the enzyme.

THE AIMS OF THE PRESENT WORK

If it is assumed that an enzymic reaction requiring

the presence of a metal ion, proceeds via an active enzyme-metal-substrate complex, then the two approaches considered above would lead to such a complex being formed by direct combination of the enzyme with the metal-nucleotide complex or by combination of the enzyme first with the free metal and then with the free nucleotide. It is possible that such a complex could also be formed by reaction of the enzyme with the free nucleotide and then the free metal ion. This may be illustrated diagrammatically:



The object of the work described in this thesis has been to use a general kinetic approach, which would consider all the possible ways in which such an EMS complex might be formed, in an attempt to define the role of the Mg^{2+} ion in the activation of the reaction catalysed by creatine kinase. Provided that all the steps, which lead to the formation of the EMS complex,

are in equilibrium, the above three pathways involve six dissociation constants, which are linked by the relationship $K_1K_2 = K_3K_4 = K_5K_6$. That is, the concentration of EMS must be the same no matter by which pathway it is formed. If one of these constants and the concentrations of the free metal and free nucleotide are known, then it is possible, from kinetic data, to assign values to all of the equilibrium constants. A full treatment of the theory of this kinetic approach, due to Professor A.G. Ogston, is reproduced in Chapter IV.

The only K value which is easily measurable is K_5, the inverse of the stability constant of the metal-nucleotide complex. This is also required to calculate the relative concentrations of free metal, free nucleotide and metal-nucleotide complex in the reaction system. A number of estimates of $^1/K_5$ have appeared in the literature (see Bock, 1960). The variation in these values is considerable, even allowing for differences in experimental parameters such as ionic strength, pH and nature of the supporting medium used for the measurements. In view of this variation, independent determinations of $^1/K_5$ for $MgATP^{2-}$ and $MgADP^-$ have been carried out under conditions so that they would be directly applicable to the enzymic kinetic studies.

CHAPTER II

THE MEASUREMENTS OF THE STABILITY CONSTANTS OF MAGNESIUM-ADENINE NUCLEOTIDE COMPLEXES

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INTRODUCTION

Various attempts have been made to determine the stability constants of $MgADP^-$ and $MgATP^{2-}$ complexes (see Bock, 1960) but there have been discrepancies in the values reported by a number of investigators (Table 1). The variation in the values for $MgADP^-$ is not as great as those for $MgATP^{2-}$ which range over an order of magnitude. This variation may be attributed in part to the different experimental techniques used. In principle, the preferred technique for measuring the stability constants of metal-ligand complexes is that of pH-titration (Rossotti and Rossotti, 1961), where the ligand is titrated in the presence and absence of a known quantity of metal ion. From the titration curves, data may be extracted to fit equations involving the formation of metal complexes.

The application of pH-titration measurements to the determination of the stability constants of metal-nucleotide complexes has proved difficult in practice because the large number of ionising groups on the

TABLE 1

Reported values of the stability constants, K, of magnesium-nucleotide complexes, where $K = \frac{[\text{Mg-nucleotide}]}{[Mg^{2+}][\text{free nucleotide}]}$.

K (M^{-1})		Method	Conditions	Reference
$MgADP^-$	$MgATP^{2-}$			
1,000	3,000	pH titration	0.2 M tetra-n-propyl-ammonium bromide, 25°	Smith and Alberty (1956)
1,300	10,000	pH titration	0.1 M KCl, 20°	Martell and Schwarzenbach (1956)
560	2,000	Ion exchange	0.085 M NaCl plus 0.02 M Tris, pH 8.0, 23°	Nanninga (1957)
1,400	11,000	Ion exchange	0.1 M NaCl plus 0.01 M Tris, pH 8.2, 23°	Walaas (1958)
2,200	38,000	Spectral changes of 8-hydroxy-quinoline	0.085 M tributylethyl-ammonium bromide plus 0.025 M triethanolamine hydrochloride, pH 8.4, 25°	Burton (1959)
-	90,000	Estimated value which fits enzyme kinetic data	0.05 M glycine-NaOH, pH 9.0, 30°	Noda, Nihei and Morales (1960)

nucleotide lead to complex mathematical expressions, which are difficult to solve. They have been simplified by the use of various approximations, but these have generally been unwarranted. Thus, Martell and Schwarzenbach (1956) assumed that in the presence of a ten-fold excess of magnesium ion, the titration curve of ATP was represented by the equation,

$$MgHATP^{-} \longrightarrow MgATP^{2-} + H^{+}$$

the concentrations of the species $HATP^{3-}$ and ATP^{4-} being considered to be negligible. Again, Smith and Alberty (1956) neglected the acid consumed in protonating the purine base when calculating the free hydrogen ion concentration of such solutions.

Other methods which have been widely used are those involving competition between ligands. A competing ligand may be a resin (Schubert, 1952) or a second organic compound whose metal complex has a different absorption spectrum to that of the free ligand. In both cases, measurements are usually carried out at constant pH and ionic strength. Thus the effect of any buffers used should be considered in terms of their possible interaction with metal ions and cognisance should be taken of the pH relative to the concentrations of the different ionised species of the nucleotide.

The possible effect of other supporting media has often been overlooked. Although values are available for the stability constants of potassium-ATP and sodium-ATP complexes (Melchior, 1954; Smith and Alberty, 1956), Martell and Schwarzenbach (1956) carried out their measurements in 0.1 M KCl and considered that the interaction of the potassium cation with ATP^{4-} was negligible. The same assumption for Na^{+} was made by Walaas (1958), who carried out ion exchange measurements in 0.1 M NaCl. Temperature and ionic strength have also been shown to affect the apparent values of the stability constants of metal-ATP complexes (Nanninga, 1957; Burton, 1959).

It is apparent that even if selection of a "correct" stability constant were possible from the values reported in Table 1, then extrapolation to other experimental conditions would be difficult.

If stability constants of the metal-adenine nucleotide complexes are required for the interpretation of enzyme kinetic experiments, it is preferable that they be determined under the conditions as close as possible to those for the kinetic measurements. Hence, determinations of stability constants have been carried out using Burton's (1959) spectrophotometric technique. In addition, a pH titration technique has been used to provide confirmation of these results and a more nearly "thermodynamic" value for the stability constant of $MgATP^{2-}$.

THEORY

The determination of stability constants using the spectral changes of 8-hydroxyquinoline.

The theory of this method has been fully elaborated by Burton (1959) and only the salient features are reproduced. Concerning, for example, ATP, it can be shown (Burton, 1959) that the stability constant, K, of $MgATP^{2-}$ is given by

$K = \frac{1}{[Mg^{2+}]_{1/2}}$ where $[Mg^{2+}]_{1/2}$ is the free magnesium concentration when $[MgATP^{2-}] = \frac{1}{2} [ATP]_T$; i.e. referring to Fig. 1,

$$K = \frac{1}{BC} \text{ at } CD = \frac{1}{2} [ATP]_T .$$

However, $[Mg^{2+}]_{1/2}$ was found by Burton to depend on the concentration of $[ATP]_T$. Thus K is represented by

$$K = \frac{1}{[Mg^{2+}]_{1/2}} + k_1 [ATP]_T$$

where k_1 is a factor introduced by Burton to allow for this dependence, attributed by him to the formation of a ternary nucleotide-metal-8-hydroxyquinoline complex. K is found by plotting values of $[Mg^{2+}]_{1/2}$ for various values of $[ATP]_T$ and extrapolating to $[ATP]_T = 0$.

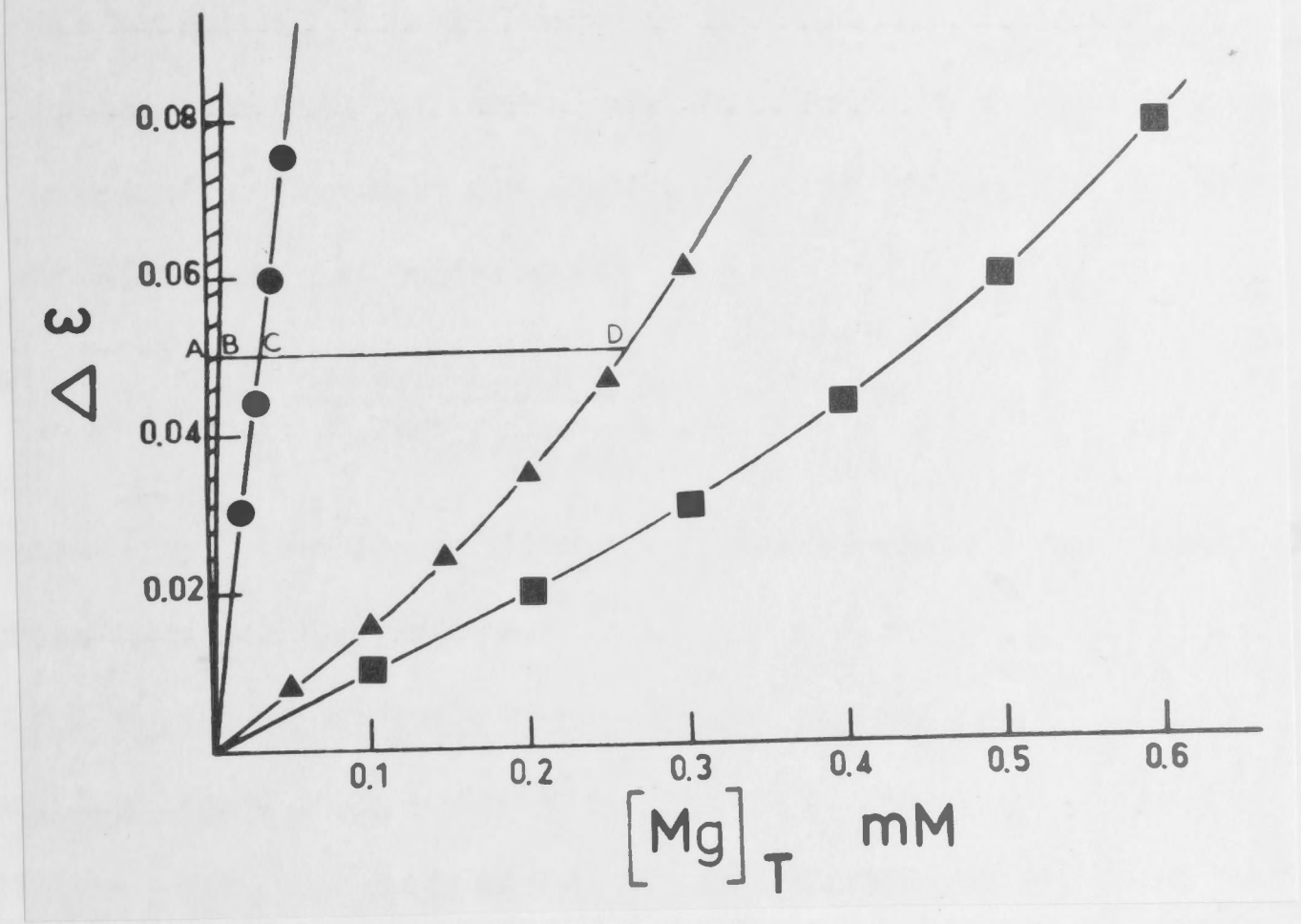

Fig. 1. Formation of $MgATP^{2-}$ complex. 0.75 mM 8-hydroxyquinoline, 0.1 M triethanolamine hydrochloride (pH 8.0); temp. 30°; cells 4 cm. ●—● , no ATP; ▲—▲ , 2 mM ATP; ■—■ , 3 mM ATP. The shaded area indicates the amount of Mg-8-hydroxyquinoline complex. For the same extinction, AB = magnesium bound to oxine, BC = free Mg^{2+}, CD = magnesium bound to ATP. $\Delta \varepsilon$ = change in extinction at 360 mμ.

An estimate of K can also be obtained from the initial slope of the titration curve for ATP. Again, the value of K was found to depend on the $[ATP]_T$ concentration, so that Burton found it necessary to introduce another constant, k_2, to allow for this. He derived the expression

$$K = \frac{[MgATP^{2-}]}{[Mg^{2+}][ATP]_T} + k_2 [ATP]_T$$

for $[Mg^{2+}] \longrightarrow 0$, to describe the slope of the initial portion of the curve.

<u>The determination of stability constants using pH titration</u>.

For the titration of the disodium salt of ATP in the presence of Mg^{2+}, the following equilibria must be considered:

$$\begin{array}{ccccc} H_2ATP^{2-} & \overset{pK_{a_1}}{\rightleftharpoons} & HATP^{3-} & \overset{pK_{a_2}}{\rightleftharpoons} & ATP^{4-} \\ \Big\Vert K_1 & & \Big\Vert K_2 & & \Big\Vert K_3 \\ MgH_2ATP & \overset{pK_{m_1}}{\rightleftharpoons} & MgHATP^{-} & \overset{pK_{m_2}}{\rightleftharpoons} & MgATP^{2-} \end{array}$$

where K_1, K_2 and K_3 are the stability constants of the appropriate magnesium complexes, with respect to dissociation into metal ion and ligand.

Linear equations describing these equilibria may be set up in terms of total ATP, total Mg and $\sum$(positive charges) = $\sum$(negative charges). These equations contain

two more unknowns than there are measurable quantities and thus are incapable of exact solution at any given titration point. Two attempts to overcome this have been made; viz. an approximation method and the use of a 1620 IBM computer to solve the equations using pairs of selected points.

A. Approximation technique.

The titration of ATP in the presence of a twenty-fold excess of Mg^{2+} was used to obtain an estimate of pK_{m_2}, which value may be used to obtain a first approximation of K_2 and K_3. In this treatment, MgH_2ATP is neglected and only the species $MgHATP^-$ and $MgATP^{2-}$ are considered. This would be reasonably valid above pH 5. For convenience, the designations L = ATP and M = Mg, are used.

First approximation.

Considering the total metal concentration,

$$[M^{2+}] + [MHL^-] + [ML^{2-}] = [M]_T \qquad (1)$$

so that $[ML^{2-}] + [MHL^-] = [M]_T - [M^{2+}]$

and hence $$[ML^{2-}]\left\{1 + \frac{[H^+]}{K_{m_2}}\right\} = [M]_T - [M^{2+}] \qquad (2)$$

Considering total ligand concentration,

$$[MHL^-] + [ML^{2-}] + [H_2L^{2-}] + [HL^{3-}] + [L^{4-}] = [L]_T \qquad (3)$$

From (1); $[M^{2+}] = [M]_T - [MHL^-] - [ML^{2-}]$

$$= [M]_T - [L]_T + [H_2L^{2-}] + [HL^{3-}] + [L^{4-}]$$

For $[M]_T = [L]_T$

$$[M^{2+}] = [H_2L^{2-}] + [HL^{3-}] + [L^{4-}] \qquad (4)$$

Considering the sum of positive and negative charges of the species in solution at any time,

$$[K^+] + [H^+] + 2[L]_T + 2[M^{2+}] = [MHL^-] + 2[ML^{2-}]$$

$$+ 2[H_2L^{2-}] + 3[HL^{3-}] + 4[L^{4-}] + [OH^-] + 2[M]_T \qquad (5)$$

(N.B. $2[L]_T$ term arises because ATP is added as the disodium salt; $2[M]_T$ term because magnesium is added as the chloride).

Substituting for $[M^{2+}]$ gives

$$[K^+] + [H^+] - [OH^-] = [MHL^-] + 2[ML^{2-}] + [HL^{3-}] + 2[L^{4-}] \qquad (6)$$

Substituting for $[MHL^-]$, $[ML^{2-}]$ and then $[M^{2+}]$ gives $[L^{4-}]$. This is used to calculate $[M^{2+}]$ and $[ML^{2-}]$ and thus K_3 from $K_3 = \dfrac{[ML^{2-}]}{[M^{2+}][L^{4-}]}$. (7)

Similarly, calculation of $[MHL^-]$ gives

$$K_2 = \frac{[MHL^-]}{[M^{2+}][HL^{3-}]} \qquad (8)$$

Once a reasonable estimate of these two constants is obtained, K_2 is utilised in a more exact theory. This makes no assumptions about pK_{m_2} but presumes that K_2 is known.

Second approximation.

Considering equations (1), (4) and (6),

$$\text{i.e., } [M^{2+}] + [MHL^{-}] + [ML^{2-}] = [M]_T = [L]_T \qquad (1)$$

$$[M^{2+}] = [H_2L^{2-}] + [HL^{3-}] + [L^{4-}] \qquad (4)$$

$$[K^+] + [H^+] - [OH^-] = [MHL^{-}] + 2[ML^{2-}] + [HL^{3-}] + 2[L^{4-}] \qquad (6)$$

Substituting successively in (6) for $[ML^{2-}]$ and $[M^{2+}]$,

$$[K^+] + [H^+] - [OH^-] = 2[M]_T - 2[M^{2+}] - [MHL^{-}] + [HL^{3-}] + 2[L^{4-}]$$

$$= 2[L]_T - [MHL^{-}] - 2[H_2L^{2-}] - [HL^{3-}]$$

Substituting for $[MHL^{-}] = K_2[M^{2+}][HL^{3-}]$ and expressing all L-terms in terms of $[L^{4-}]$,

$$[L^{4-}]\, K_2\frac{[H^+]}{K_{a_2}}\left\{1 + \frac{[H^+]}{K_{a_2}} + \frac{[H^+]^2}{K_{a_1}K_{a_2}}\right\} + [L^{4-}]\frac{[H^+]}{K_{a_2}}\left\{1 + \frac{[H^+]}{K_{a_2}}\right\} =$$

$$2[L]_T - [K^+] - [H^+] + [OH^-]$$

$$\text{i.e., } [L^{4-}] = -\frac{B + \sqrt{B^2 + 4AC}}{2A} \qquad (9)$$

where $A = K_2\frac{[H^+]}{K_{a_2}} \cdot \left\{1 + \frac{[H^+]}{K_{a_2}} + \frac{[H^+]^2}{K_{a_1}K_{a_2}}\right\}$

$$B = \frac{[H^+]}{K_{a_2}} + \frac{2[H^+]^2}{K_{a_1}K_{a_2}}$$

$$C = 2[L]_T - [K^+] - [H^+] + [OH^-] .$$

The ratio of bound to total metal is given by

$$\bar{n} = \frac{[MHL^-] + [ML^{2-}]}{[M^{2+}] + [MHL^-] + [ML^{2-}]} \qquad (10)$$

$$= \frac{[L]_T - [L^{4-}]\left\{1 + \frac{[H^+]}{K_{a_2}} + \frac{[H^+]^2}{K_{a_1}K_{a_2}}\right\}}{[M]_T} \qquad (11)$$

Equation (10) may also be expressed in the form,

$$\bar{n} = \frac{K_2[HL^{3-}] + K_3[L^{4-}]}{1 + K_2[HL^{3-}] + K_3[L^{4-}]} \qquad (12)$$

Rearranging: $$K_3 = \frac{\bar{n}}{(1 - \bar{n})} \cdot \frac{1}{[L^{4-}]} - K_2 \cdot \frac{[HL^{3-}]}{[L^{4-}]} \qquad (13)$$

B. <u>Analysis of data by the 1620 IBM Computer</u>.

The same basic equations, describing the equilibria between the different species of ATP and the corresponding magnesium complexes, as for the approximation method were used, except that K_1 (the stability constant of MgH_2ATP) was included. The rationale of the method was to select different values of K_1 and to compute the best values for K_2 and K_3 from pairs of points selected along the titration curve. Thus for the two points considered, different values of K_2 and K_3 were obtained for each value of K_1 and it was possible, by comparing the results from different pairs of points, to select the "best" value for each constant, or at least to define the limits within which those constants must lie.

The author's contribution to this work was minor as the major part of the theoretical work was developed by Dr. D. D. Perrin and the Programme was written by Miss E. A. Reid. Thus, full details of the theory and the logic used, are included in this thesis as an appendix.

MATERIALS

Nucleotides.

ATP, obtained as the disodium salt from Sigma Chemical Co., was twice recrystallised from 50% ethanol (Berger, 1956), dried in air at room temperature and stored at -10°. The resultant salt was characterised as $Na_2ATP.4H_2O$ by elementary analysis (found: P, 14.9; N, 11.27; H_2O, 10.9 : theory for $Na_2ATP.4H_2O$; P, 14.9; N, 11.24; H_2O, 11.5%) and by determination of the molecular extinction at 259 mμ (Bock, Ling, Morell and Lipton, 1956). No ultraviolet-light-absorbing contaminants were observed after chromatography in isobutyric acid-NH_3 (sp.gr., 0.91)-water (66:1:33. v/v) (Krebs and Hems, 1953).

ADP, obtained as a Na-salt from Sigma Chemical Co., was subjected to paper chromatographic analysis under the same conditions as described above. Examination in ultraviolet light showed that all samples were contaminated with AMP and further purification was carried out.

ADP (1.7 g.) was dissolved in 50 ml. of water, the solution adjusted to pH 7.6 with 1 N NaOH, diluted to 400 ml. and passed through a column (2.5 x 9.5 cm.) of Dowex-1 (formate, 200-400 mesh). The column was washed with increasing concentrations of formic acid (0.25 - 2.0 M) before the ADP was eluted with 4 M formic acid. The ADP was adsorbed onto a column of Nuchar C (8 g.), which was washed with water, and eluted with ethanol-NH_3 (S.G., 0.88)- water (40:0.1:60, by vol.). The barium salt of ADP was precipitated by the addition of 75 ml. of 1.5% (w/v) barium bromide in 50% (v/v) ethanol and 120 ml. of absolute ethanol to 580 ml. of eluate cooled to 0°. After 15 min. the precipitate was collected by centrifugation at 0°, washed with ethanol and ether and dried in a vacuum desiccator. The barium salt of ADP was converted to the sodium salt by adding it as a slurry to the top of a column (1 x 15 cm.) of Zeo-Karb 225 (Na^{+}) and washing through with water. The solution of NaADP (pH 7.0) was free from barium and did not contain any impurities that absorbed ultraviolet light as judged by paper chromatographic analysis as described above. Following the determination of the ADP concentration by measurement at 259 mμ (Bock, et al., 1956), the solution was diluted to a concentration of 10^{-2}M and stored at -10°.

$MgCl_2$.

An aqueous solution of $MgCl_2.6H_2O$ (AnalaR) was standardised by passing measured amounts through an Amberlite IR-120 (H^+) column and titrating the effluent acid with standard alkali.

Supporting medium.

Tetraethylammonium bromide, obtained as B.D.H. Laboratory Reagent was twice recrystallised from alcohol-ether.

Carbonate-free alkali.

Carbonate ion was precipitated from a stock solution of KOH (approximately 1 N prepared from AnalaR reagent) with barium hydroxide solution and the excess barium was removed by passing the alkali through a column of Amberlite IR-120 (K^+ form). The alkali was collected under nitrogen, standardised, and diluted as required with carbonate-free water.

Buffers.

Commercial triethanolamine was purified, following the method of Germann and Knight (1933).

Approximately 200 ml. of crude triethanolamine was neutralised to a slight excess of acid, with 10 N HCl, the temperature being maintained below 15°. The crystals of triethanolamine hydrochloride were allowed to settle for one hour, washed thoroughly with 95% ethanol and dried

for an hour at 110°. The yield of the hydrochloride was 192 g. KOH pellets (44 g.) were ground under isopropyl alcohol (100 ml.) in a mortar and added to 800 ml. of isopropyl alcohol. Triethanolamine hydrochloride was then added, the mixture refluxed in a water bath for 3 hr., allowed to stand overnight and the precipitated NaCl removed by filtration. The isopropyl alcohol was removed by distillation at atmospheric pressure. Triethanolamine was obtained by distilling under reduced pressure (0.5 mm.Hg) and that fraction distilling at 167-168° was collected. Nitrogen analysis indicated that the purified preparation was 98-99% pure.

Tris(hydroxymethyl)aminomethane (TRIS) was a Fisher Certified Reagent issued as 99.98% pure.

N-ethylmorpholine, as obtained from Eastman-Kodak was distilled three times under reduced pressure at 30°.

The buffers were prepared at 0.5 M concentration, adjusted to the required pH by the addition of 5 N HCl and diluted as required. The pH values of the diluted buffer solutions were checked on a Vibron Electrometer Model 33B pH meter reading to 0.001 of a pH unit.

8-Hydroxyquinoline was AnalaR reagent grade material.

Phosphorylcreatine.

PC was prepared by the method of Ennor and Stocken (1948). The crystalline product (5 g.) was dissolved

in 100 ml. of water, passed through a column (1.5 x 10 cm.) of Dowex-50 (Na^+, 200-400 mesh) and recrystallised from the effluent (150 ml.) by the addition of absolute ethanol to a concentration of 80% (v/v). Elementary analysis indicated that the product was pure and contained 6 moles of water of crystallisation.

METHODS

The determination of stability constants using the spectral changes of 8-hydroxyquinoline.

The concentration of magnesium-8-hydroxyquinoline complex formed was determined by carrying out measurements at 360 mμ., the wavelength of maximum spectral difference between 8-hydroxyquinoline and its magnesium complex, on a Shimadzu (QR-50) spectrophotometer. $MgCl_2$ (0.1 M) was added from an Agla Micrometer Syringe to the solution of 8-hydroxyquinoline in 0.1 M buffer maintained at 30°. The pH of the solution was checked on the Vibron Electrometer pH meter.

The stability constant of $MgATP^{2-}$ was determined by carrying out spectral measurements in 10.0 ml. of solution contained in a cell of 4 cm. light path and with 8-hydroxyquinoline at concentrations of 0.3, 0.4 or 0.75 mM. The measurements for $MgADP^-$ were carried out in 3.0 ml. of solution in a cell of 1 cm. light path and with

8-hydroxyquinoline at a concentration of 0.4 mM. The molar extinction of the Mg-8-hydroxyquinoline complex was found by measuring the absorption of 0.1 mM 8-hydroxyquinoline in 0.08 M $MgCl_2$. A plot of the change in extinction at 360 mμ ($\Delta\varepsilon$) against added $MgCl_2$, in the absence of ATP and in the presence of two concentrations of ATP, is shown in Fig. 1.

<u>The determination of the stability constants of magnesium adenine nucleotide complexes by pH-titration</u>.

The two terminal acid dissociation constants of ATP were determined by titrating a 5.25×10^{-4} M solution of the disodium salt of ATP at 30° with KOH (0.105 N). The titration was carried out in 50 ml. of solution in a 200 ml. lipless Pyrex beaker closed by a rubber stopper through which passed the electrodes, a thermometer, a bubbler and a fine-bore polythene tube, which was connected to an Agla micrometer syringe. The solution was stirred by passing scrubbed nitrogen through the bubbler. pH measurements were made using the Vibron Electrometer Model 33B (Electronic Instruments Ltd.) fitted with an internally shielded glass electrode. The output of the pH meter was recorded on a Rectiriter recording milliammeter (Texas Instruments Inc.) to facilitate assessment of attainment of equilibrium. Calibration was based on the standards, potassium hydrogen

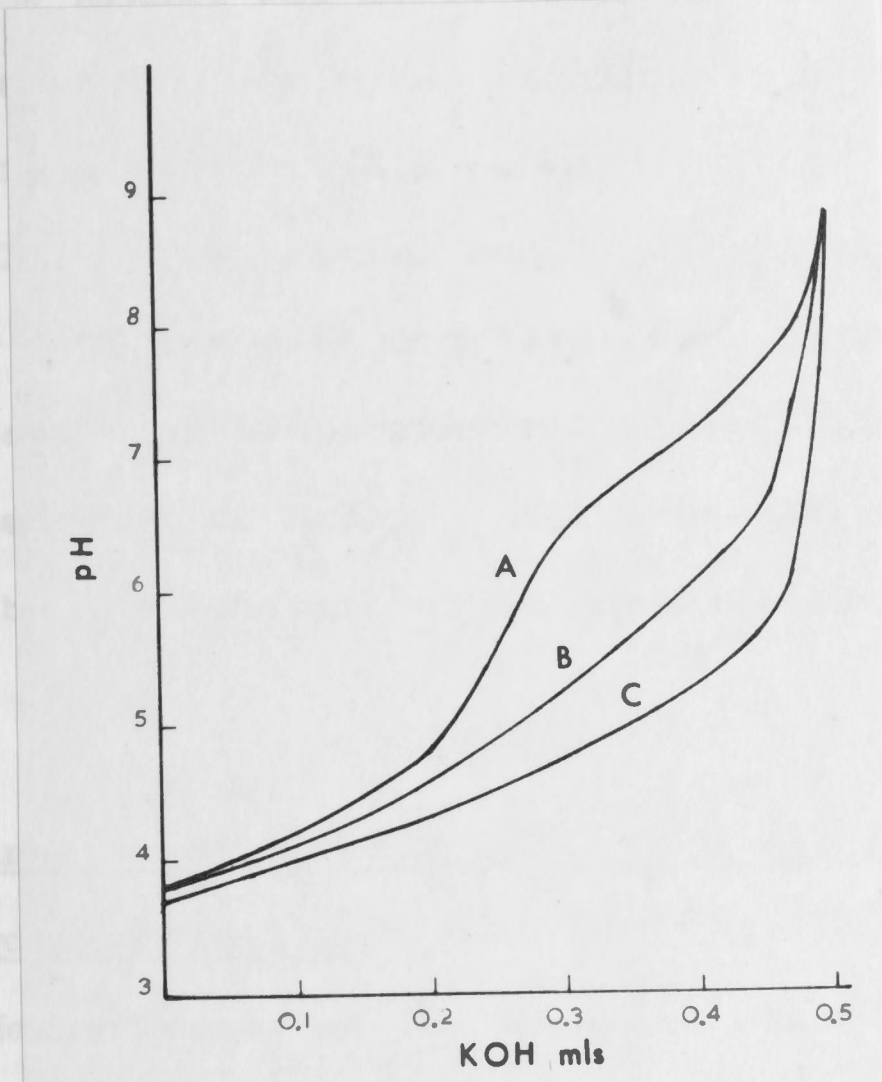

Fig.2. Titration curves of ATP. All curves, $[ATP]_T = 5.25 \times 10^{-4}M$. Curve A, $[Mg]_T = 0$; curve B, $[Mg]_T = 5.25 \times 10^{-4}M$ ($[Mg]_T = [ATP]_T$); curve C, $[Mg]_T = 1.05 \times 10^{-2}M$ ($[Mg]_T = 20[ATP]_T$). Titrations carried out in 50 ml. of solution, ionic strength = 0.1, temp. 30°.

phthalate (0.05 M), pH_{30^o} = 4.011 and sodium tetraborate (0.05 M), pH_{30^o} = 9.142 (Perrin, 1960).

The alkali was added from the micrometer syringe, a volume of 0.5 ml. being required to complete the titration. pH readings were usually taken after the addition of 0.01 ml. of alkali. The initial and final pH values were approximately 3.75 and 8.8, respectively. ATP was titrated in the presence of two concentrations of $MgCl_2$, as well as in the absence of $MgCl_2$. The ionic strength was maintained at 0.1 by the addition of tetraethylammonium bromide.

RESULTS

Determination of K for $MgATP^{2-}$ from the spectral changes of 8-hydroxyquinoline.

Measurements of the apparent stability constant (K) of $MgATP^{2-}$ at pH 8.0 have been carried out in three buffers, tris(hydroxymethyl)aminomethane (TRIS), triethanolamine and N-ethylmorpholine. The results are shown in Table 2, together with an "absolute" value, K*, calculated on the basis that the pK_a of the final phosphate group of ATP is 7.0 and thus at pH 8.0, approximately 90% of the ATP is present as ATP^{4-}. Similar results were obtained at the three concentrations, 0.3, 0.4 and 0.75 mM, of 8-hydroxyquinoline used.

The nature of plot from which the value of K is determined is illustrated in Fig.3 where points for

TABLE 2

Measured values for the stability constant of $MgATP^{2-}$

(K refers to the experimental stability constant and K* to an "absolute" stability constant calculated from K on the basis that the pK_a of the terminal group of ATP was 7.0).

Method	pH	K (M^{-1})	K* (M^{-1})	No. of experiments
Spectral changes of 8-hydroxyquinoline:				
(a) TRIS buffer	7.99	18,000 ±2,000	20,000	3
(b) Triethanolamine buffer	8.02	70,000 ±6,000	77,000	4
(c) N-Ethylmorpholine buffer	8.01	77,000 ±7,000	85,000	3

pH titration

(a) Approximation method: 90,000 M^{-1}

(b) Value obtained from computer: 75,000 M^{-1}

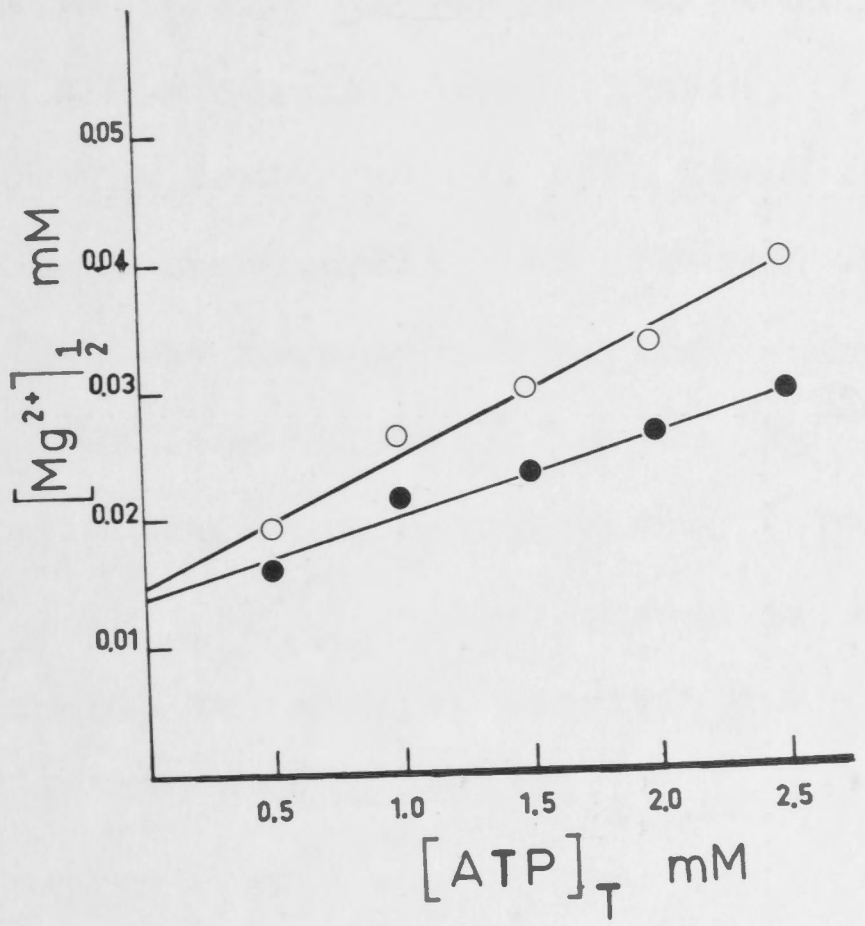

Fig. 3. Determination of the stability constant of $MgATP^{2-}$ in triethanolamine buffer (0.1 M, pH 8.0) at 30°. ●—●, $[Mg^{2+}]_{\frac{1}{2}}$ values; O - O, initial slope values. The $[Mg^{2+}]_{\frac{1}{2}}$ values extrapolate to 0.014 mM so that K = 71,400.

$[Mg^{2+}]_{\frac{1}{2}}$ and for the initial slopes are shown. Greater weight has been placed on the $[Mg^{2+}]_{\frac{1}{2}}$ points as it was possible to determine these with greater precision.

The variation in the value of $[Mg^{2+}]_{\frac{1}{2}}$ with the concentration of ATP, as determined in different experiments, in the presence of triethanolamine, is illustrated in Fig. 4. The best line has been drawn using the average value for each concentration of ATP used and the variation in the values, at each concentration is indicated. It was found that less variation in the $[Mg^{2+}]_{\frac{1}{2}}$ values was obtained with lower concentrations of $[ATP]_T$. Above 3mM $[ATP]_T$, the $[Mg^{2+}]_{\frac{1}{2}}$ values tended in some cases to deviate towards the x-axis, possibly due to the formation of the complex $Mg(ATP)_2^{6-}$.

An estimate of K was carried out in 0.02 M N-ethylmorpholine, while the ionic strength was kept constant at 0.1 by the addition of tetraethylammonium bromide. The value obtained was 80,000 M^{-1}, corresponding to a K^* of 88,000 M^{-1}. This is greater than the value obtained in 0.1 M N-ethylmorpholine but the variation is within the experimental error.

Determination of K for $MgADP^-$.

A plot of $[Mg^{2+}]_{\frac{1}{2}}$ and initial slope values against $[ADP]_T$ is shown in Fig. 5, giving an estimate of 4,150 M^{-1} for the stability constant of $MgADP^-$. A repeat experiment

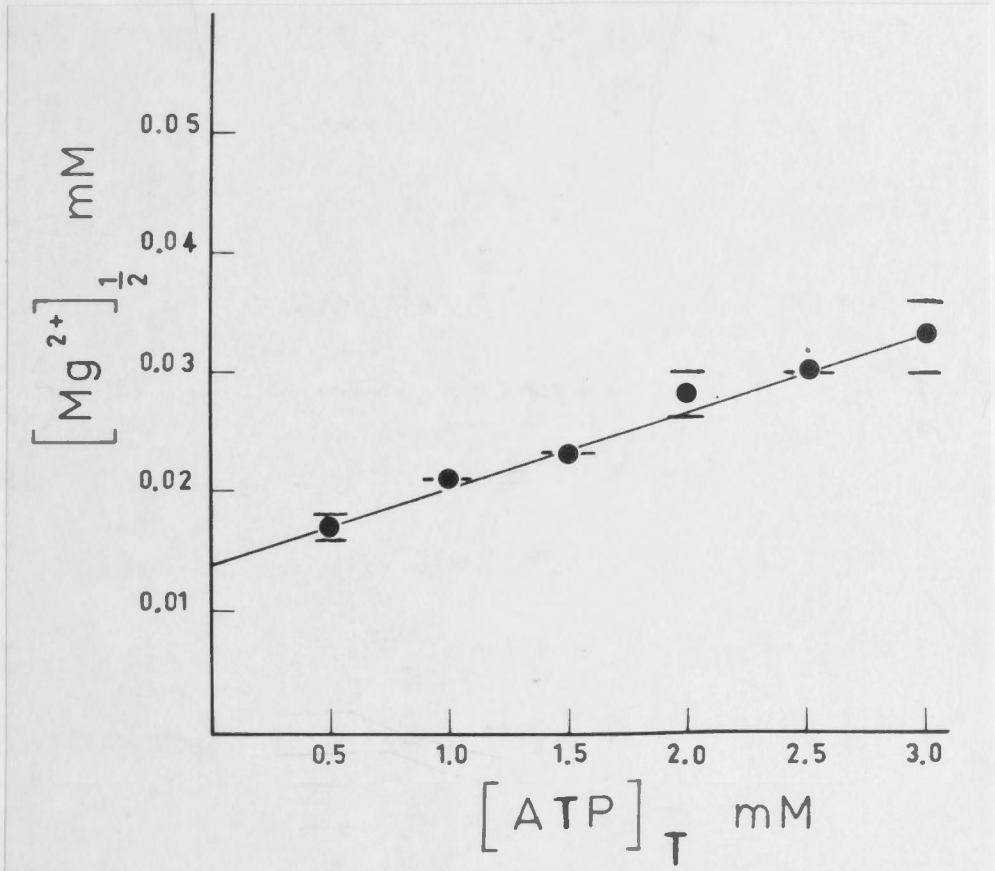

Fig. 4. Determination of the stability constant of $MgATP^{2-}$. Variation in the values of $[Mg^{2+}]_{\frac{1}{2}}$ in triethanolamine buffer (0.1 M, pH 8.0) at 30°. The line extrapolates to $[Mg^{2+}]_{\frac{1}{2}}$ = 0.014 mM so that K = 71,400.

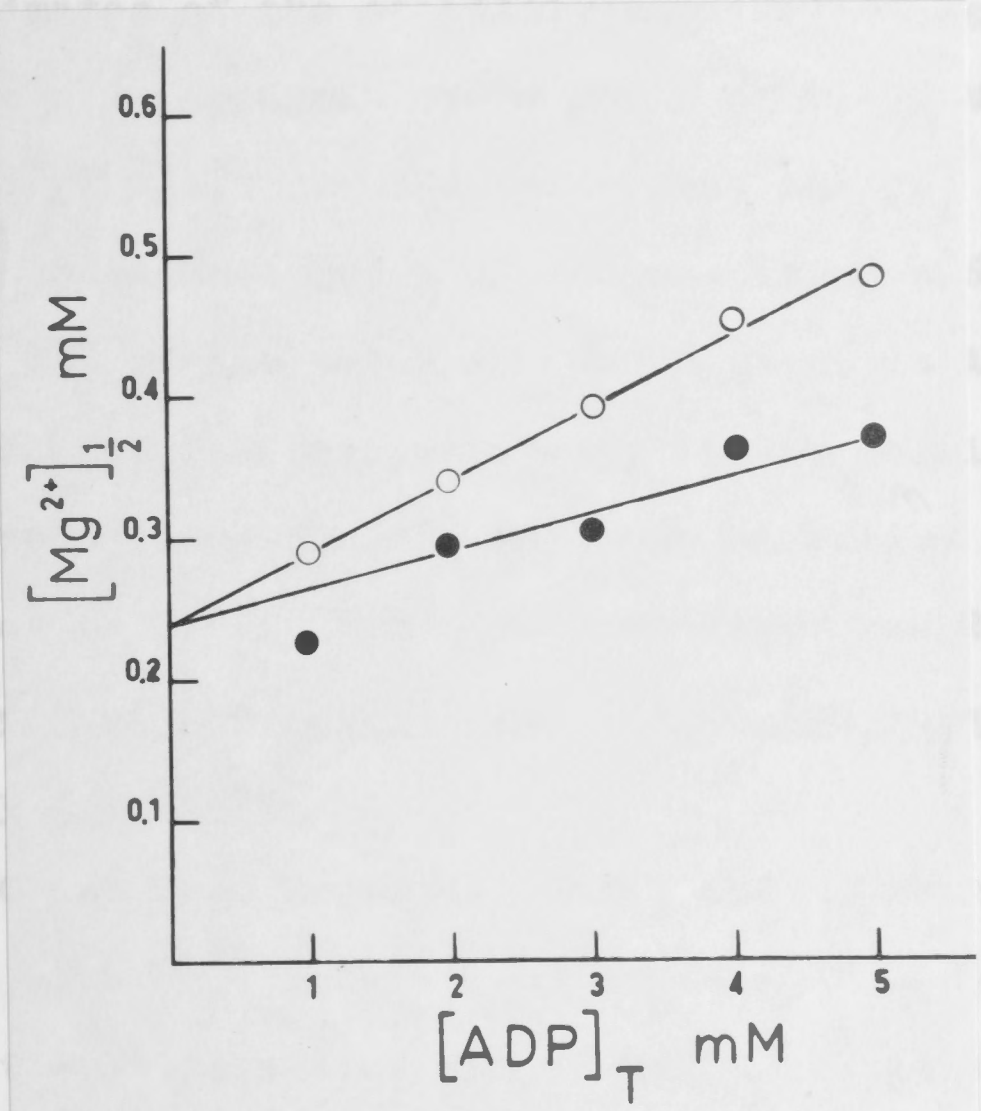

Fig. 5. Determination of the stability constant of $MgADP^-$ in N-ethylmorpholine buffer (0.1 M, pH 8.0) at 30°. ● - ● , $[Mg^{2+}]_{\frac{1}{2}}$ values; O - O, initial slope values. Lines extrapolate to 0.24 mM so that $K = 4,150\ M^{-1}$.

gave a value of 4,000 M^{-1}.

The amount of complex formation between Mg^{2+} and 8-hydroxyquinoline at pH 7.0 was found to be too small for estimates of the stability constant of $MgADP^-$ at this pH. An apparent value for K of 2,400 M^{-1} was found at pH 7.4. On the basis that the pK_a for the terminal phosphate group of ADP was at pH 6.65, and thus 70% and 85% of the total ADP was present as ADP^{3-} at pH 7.0 and pH 7.4, respectively, it was possible to calculate a value for the apparent stability constant of $MgADP^-$ at pH 7.0. The figure obtained was 2,000 M^{-1}.

<u>The effect of phosphoryl creatine on the apparent stability constant for $MgADP^-$</u>.

PC has been reported (Smith and Alberty, 1956) to complex with Mg^{2+} and is thus capable of affecting the apparent stability constant of $MgADP^-$. As the enzyme experiments were to be carried out in the presence of PC concentrations in the range 5 to 20 mM, the effect of these concentrations of PC on the apparent stability constant of $MgADP^-$ was determined.

It was found that the apparent K value for $MgADP^-$ decreased with increasing concentrations of PC (Table 3). Some difficulty was found in obtaining the apparent K value for PC at a concentration of 10 mM, though not at 20 mM, as there was considerable variation in the $[Mg^{2+}]_{\frac{1}{2}}$

TABLE 3

The effect of PC concentration on the apparent stability constant, K, of $MgADP^-$. Estimates carried out at 30° in 0.1 M N-ethylmorpholine buffer, pH 8.0.

PC Concentration (mM)	K (M^{-1})	$^1/K$ (mM)
-	4,000	0.25
10	3,600	0.28
20	3,300	0.30

$$K(MgPC) = 12\ M^{-1}$$

values. However, it was possible to take a direct ratio of the $[Mg^{2+}]_{\frac{1}{2}}$ quantity, with and without PC, at each concentration of ADP used.

By multiplying the average ratio by the value of K in the absence of PC, it was possible to obtain an estimate of the apparent K at PC = 10 mM.

A stability constant value of 12 M^{-1} for MgPC was calculated from the expression

$$K = K_{app.} (1 + K_{PC}[PC]).$$

This may be compared with the value of 20 M^{-1} reported by Smith and Alberty (1956).

<u>The determination of stability constants from the pH-titration data.</u>

Titration of ATP under the specified conditions gave pK_a values, calculated from the complete Henderson-Hasselbach equation, of

$$pK_{a_1} \quad 3.93 \pm 0.02 \quad \text{and}$$

$$pK_{a_2} \quad 6.97 \pm 0.02 .$$

An estimate of pK_{m_2} as 4.88 was obtained from the titration with $[Mg]_T = 20[ATP]_T$.

A. <u>Calculations of stability constants by approximations.</u>

In applying the approximation techniques to a particular titration curve, 6 to 8 points were selected between pH 5.0 and pH 6.5. This pH range was chosen as

below pH 5.0, the concentration of MgH_2ATP becomes increasingly important, while above pH 6.5, the titration curve changed too rapidly with increasing concentration of alkali for accurate determinations to be made. The first approximation method, dependent on the value of pK_{m_2}, was used to give a series of values for K_2 and K_3, which are pH dependent. These values, plotted against pH, give a parabola which may face up or down as illustrated for K_3 in Fig. 6. The values of K_2 and K_3, obtained from the maximum or minimum of such parabolas, were taken as representing the first approximation.

In this way, different titration curves have given values for K_2 and K_3 from 500 to 850 M^{-1} and from 70,000 to 107,000 M^{-1} respectively.

An estimate of K_2, within the above range was then used in the second approximation. The results obtained were compared with results using a value of K_2 slightly above or slightly below the first estimate. This either flattened out the parabola (e.g. EF in Fig. 6) or increased its curvature. The "best" estimate of K_3, and of K_2, was taken as being given by those values that gave the parabola of least curvature.

The "best" estimates of K_2 and K_3, obtained in this way from separate experiments, are shown in Table 4, as are also the average values. These may be compared with

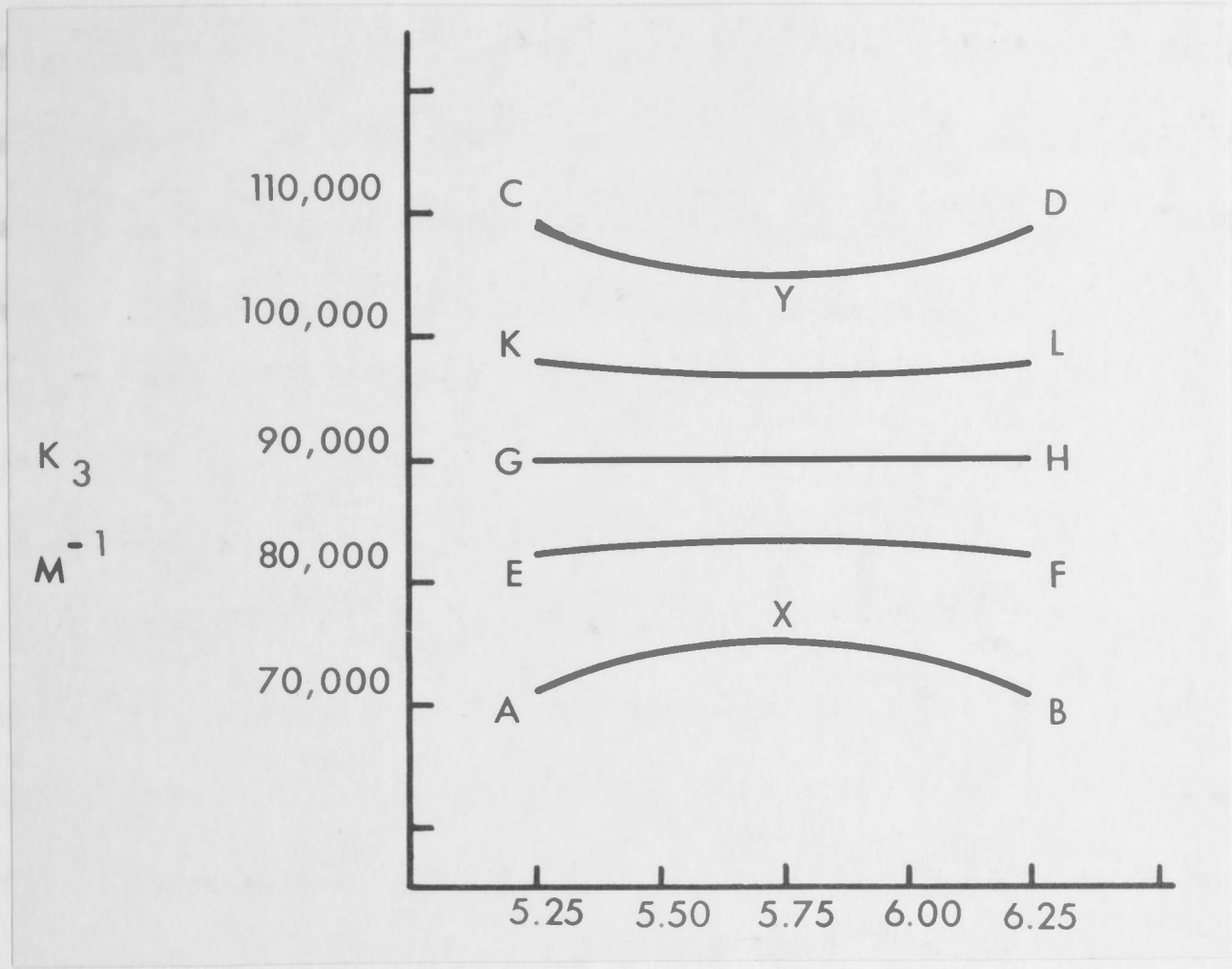

Fig. 6. Graphical representation of the approximation methods used to determine the stability constant of $MgATP^{2-}$. Curves AB and CD correspond to the values obtained by the first approximation method for two independent titration curves, X and Y being taken as the best values respectively. EF and KL correspond to the application of the second approximation method, leading to the "best" value of K_3 represented by GH.

TABLE 4

Separate estimates of K_2 and K_3, the stability constants of $MgHATP^-$ and $MgATP^{2-}$ respectively, as obtained by the approximation methods described in the text. Each value represents a separate experiment.

	K_2 (M^{-1})	K_3 (M^{-1})
	680	83,000
	720	97,000
	700	91,000
	750	93,000
	750	80,000
	550	75,000
	800	104,000
Average:	700	88,000

the values previously reported, of 800 M^{-1} and 105,000 M^{-1} (O'Sullivan and Perrin, 1961). The latter have since been corrected for a small systematic error, introduced by using an erroneous value for the activity coefficient of the hydrogen ion in solution.

B. The calculation of stability constants using the 1620 IBM Computer.

Only one set of titration data, for which $[Mg]_T = [ATP]_T = 5.25 \times 10^{-4}$ M, has been analysed. The titration curve was constructed from 50 individual pH readings, each obtained from the addition of 0.01 ml. of alkali to the ATP solution. Pairs of points, at intervals of 10 additions along this curve were selected for the computations, within the range pH 4.0 to pH 6.6. Points below pH 4.0 and above pH 6.6 were neglected as it was considered that these would be liable to the greatest experimental error.

The best estimates of the stability constants obtained were:

K_1 10 - 20 M^{-1}

K_2 approximately 500 M^{-1}

K_3 approximately 75,000 M^{-1}.

The effect of the assigned magnitude of K_1 on the values obtained for K_2 and K_3 and a summary of values

obtained is shown in Table 5. The positions of the pairs of points taken is also indicated.

The stability constants of other metal-nucleotide complexes.

Because creatine kinase is activated not only by Mg^{2+} but also by Ca^{2+}, Mn^{2+} and Co^{2+}, it had been hoped to carry out comparative studies with these divalent metal ions. While this has not yet been done some preliminary estimates of the metal-ADP and metal-ATP complexes have been carried out (Table 6).

The approximation methods described above were applied to data from the titration of ATP with two concentrations of $CaCl_2$ ($[Ca]_T = [ATP]_T$ and $[Ca]_T = 20[ATP]_T$ respectively) to extract a value for the stability constant of $CaATP^{2-}$.

The estimates for $CaADP^-$ and $MnADP^-$ were obtained by Burton's (1959) spectrophotometric technique, except that in the case of Mn^{2+}, catechol-3,5-disulphonic acid (disodium salt) was used instead of 8-hydroxyquinoline. Under the experimental conditions Mn^{2+} formed a precipitate with 8-hydroxyquinoline.

In conjunction with Dr. M. E. Winfield of the Division of Physical Chemistry, C.S.I.R.O., Melbourne, an attempt was made to measure the stability constant of $MnADP^-$ using an electron resonance technique. It was noted that the amplitude of the signal due to Mn^{2+} was

TABLE 5

Summary of some representative values obtained from the 1620 IBM Computer for the stability constants, K_1, K_2 and K_3 for the complexes MgH_2ATP, $MgHATP^-$ and $MgATP^{2-}$. All values of K_1, K_2 and K_3 expressed as M^{-1}.

K_1	K_2	K_3	Points used*
Zero	400	73,500	25, 15
	570	74,200	30, 20
	480	78,200	36, 26
	420	75,800	41, 31
20	400	74,200	25, 15
50	100	71,900	36, 26

*Titration curve constructed from 50 pH readings, each representing the addition of 0.01 ml. alkali.

TABLE 6

Stability constants of other metal-nucleotide complexes

Complex	Method	Conditions	K (M^{-1})
$CaATP^{2-}$	pH titration	0.1 M $(C_2H_5)_4NBr$, 30°	45,000
$CaADP^{-}$	Spectral changes of 8-hydroxy-quinoline	0.1 M N-ethyl-morpholine (pH 8.0), 30°	2,200
$MnADP^{-}$	Spectral changes of catechol-3,5-disulphonic acid	0.1 M N-ethyl-morpholine (pH 8.0), 30°	25,000
$MnADP^{-}$	Electron para-magnetic resonance	0.1 M N-ethyl-morpholine (pH 8.0), 27°	30,000

reduced on the addition of ADP. As $MnADP^-$ would have been expected to be formed under the experimental conditions, this reduction in signal was taken as a measure of the concentration of this complex and was thus used to obtain an estimate of the stability constant. However, there is considerable uncertainty about this figure as it appears that more than one complex is formed (Winfield, personal communication).

DISCUSSION

The primary object of this work was to obtain reliable values for the stability constants of metal-nucleotide complexes, which could be used in conjunction with enzyme kinetic experiments. It would appear that the procedure of Burton (1959) is suitable for this purpose since the values of K^* for $MgATP^{2-}$ in both triethanolamine and N-ethylmorpholine are in satisfactory agreement with those obtained from titration data. The analysis of the latter presents difficulties, because of the complexity of the equations used, but this has been overcome by the use of a 1620 IBM Computer.

As the differences obtained for K in triethanolamine and N-ethylmorpholine are insignificant, being within the experimental error, it may be concluded that neither buffer interacts appreciably with Mg^{2+}. On the other

hand, it is clear that TRIS does interact with Mg^{2+} (Table 2), though it has been claimed that the binding of Mg^{2+} and Ca^{2+} to this buffer may be neglected (Nanninga, 1957).

Though larger than any values previously reported, the results are of the same magnitude as Burton's (1959). If his value for $MgATP^{2-}$ is extrapolated to the experimental conditions reported above (using Burton's temperature coefficient and ignoring the slight difference in ionic strength) a value of approximately 45,000 M^{-1} is obtained, which may be compared to 70,000 M^{-1} obtained in this study. This value of 70,000 M^{-1} may also be compared with the value of 90,000 M^{-1} predicted by Noda, Nihei and Morales (1960) to fit kinetic results obtained with creatine kinase in 0.05 M glycine-NaOH at 30°, pH 9.0. Watanabe (1962) has reported a value for $MgATP^{2-}$ of the same magnitude as those reported here but did not give any figures. Two other determinations of the stability constant of $MgATP^{2-}$ have been reported recently by Nanninga (1961) and by Taqui Khan and Martell (1962) as 24,500 M^{-1} and 16,500 M^{-1} respectively. Nanninga obtained consistent results from two methods. One involved measuring the shift in pH when Mg^{2+} was added to ATP at pH 6.92. The concentration of the species $MgHATP^{-}$ was neglected, though this cannot be considered

valid at pH values below 7. The other was a resin method carried out in the presence of 0.02 M TRIS buffer. This concentration of TRIS may not have had a great effect on the value obtained but criticism has been levelled at the use of resins to determine stability constants (see Bock, 1960). The values reported by Taqui Khan and Martell may also be criticised as these were obtained from measurements in 0.1 M KNO_3, and no attempt was made to correct the values for the formation of a potassium-ATP^{3-} complex (Melchior, 1954; Bock, 1960).

The slight deviation of the $[Mg^{2+}]_{\frac{1}{2}}$ values towards the x-axis for ATP concentrations above 3 mM (see Results) suggests that small amounts of $Mg(ATP)_2^{6-}$ may form under these conditions. The precision with the ADP measurements was not great enough to detect any possible formation of $Mg(ADP)_2^{4-}$. Also, no conclusions as to the formation of Mg_2ATP or Mg_2ADP^{+} could be reached as experiments were not carried out at sufficiently high concentrations of Mg^{2+}. Confirmation of the formation of such complexes, and evaluation of their stability constants, would be theoretically possible from the titration data with $[Mg]_T = 20[ATP]_T$, but the calculations have not been attempted. Some values for the stability constants of complexes other than 1:1 have been reported but they are not well substantiated (see Bock, 1960).

Lowenstein (1960) has invoked the presence of complexes of the type M_2ATP to explain the non-enzymic hydrolysis of ATP.

Titrations of ADP in the presence of various amounts of magnesium ion have been carried out but difficulty has been experienced in interpreting the curves obtained, as it has not been possible to obtain ADP in an unequivocal salt form, and no valid estimates of the stability constant of $MgADP^-$ have been obtained by this technique. Therefore, only results from the spectrophotometric determination for the stability constant of $MgADP^-$ have been reported. The K for $MgADP^-$ is affected by concentrations of PC in the range from 5 to 10 mM.

In agreement with Burton (1959), and to a lesser extent Martell and Schwarzenbach (1956) and Walaas (1958), the results indicate a difference of an order of magnitude between the stability constants of $MgADP^-$ and $MgATP^{2-}$. The reason for this is not clear, particularly as the nuclear magnetic resonance results of Cohn and Hughes (1962) indicate that only the two terminal phosphate groups, in each case, are concerned with the binding of the metal ion.

There is still some doubt about the exact structure of the $MgATP^{2-}$ complex. It was suggested by Szent-György (1956) that the metal ion might combine not only

with the phosphate-oxyanion groups (see Cohn and Hughes, 1962) but also through the 6-amino group and the nitrogen atom at position 7 of the purine ring to form a quadridentate chelate. But the formation of such a chelate, involving a fourteen-membered ring, must be considered to involve a very unfavourable entropy change (Martell and Calvin, 1952). Rotatory dispersion measurements (Levendahl and James, 1956) and infrared studies (Epp, Ramasarma and Wetter, 1958) have been invoked as evidence in favour of such a "curled" configuration for $MgATP^{2-}$.

Martell and Schwarzenbach (1956) and Smith and Alberty (1956) did not detect any lowering of the purine pK_a in the presence of Mg^{2+} and concluded from this that Mg^{2+} did not interact with the purine ring. Walaas (1958), who carried out comparative studies with different nucleotides also concluded that there was no interaction with the purine ring as the values of the stability constants were not affected by the nature of the heterocyclic base.

Spectral shifts in the ultraviolet region were observed by Hotta, Brahms and Morales (1961) for ATP in the presence of alkali. These were similar to those observed by Bock, Ling, Morell and Lipton (1956) on the addition of alkali to ATP, and were attributed to the

displacement of protons from the adenine ring. This finding was considered by Hotta et al. (1961) to provide evidence in favour of $MgATP^{2-}$ in solution existing at least partly in a "curled" configuration.

Such conclusions are not in agreement with the nuclear magnetic resonance studies of Hammes, Maciel and Waugh (1961), who found that the signals from the ring hydrogens were unaffected by the presence of alkaline earth metal ions. This has been confirmed by phosphorus and proton magnetic resonance studies carried out by Cohn and Hughes (1962). Mg^{2+} and Ca^{2+} affected only the β- and γ-phosphate groups of ATP and did not interact with the adenine ring. By comparison, Zn^{2+}, Cu^{2+} and Mn^{2+} formed a complex with the adenine ring, involving the nitrogen at position 7, though it was pointed out that this could be due to the formation of an intermolecular complex rather than an intramolecular chelate. These workers considered that the complexing of Mg^{2+} with the β- and γ-phosphate groups could lower the pK_a of the ring sufficiently to account for the spectral shifts observed by Hotta et al. (1961). This is consistent with the present work as it was found that the presence of Mg^{2+} lowered the pK_a of the purine moiety by as much as 0.1 of a pH unit when $[Mg]_T = 20[ATP]_T$.

Brintzinger (1961) has suggested that divalent metal ions form an ion-pair association with ATP. This interpretation was based largely on the finding that the stability constants of ATP complexes with the transition metal ions, Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}, varied only slightly and did not follow the Irving-Williams series. A similar phenomenon is observed for the stability constants of the complexes formed with SO_4^{2-} by these metals and evidence has been presented that such complexes are probably ion-pairs (Smithson and Williams, 1958; Nancollas, 1960).

Indirect evidence that the complexes between Mg^{2+} and Ca^{2+} and ATP are not ion pair associations is furnished by the work of Diebler, Eigen and Hammes (1960) who used relaxation spectrometry to measure the rate of formation of $MgATP^{2-}$ and $CaATP^{2-}$. They found that, although there was little difference in the stability constants of these two complexes, $CaATP^{2-}$ was formed nearly 100 times faster than $MgATP^{2-}$. This was attributed to the fact that the rate controlling step in complex formation was the dissociation of a water molecule from the inner hydration shell of the metal ion, which could be more easily accomplished with the larger Ca^{2+} ion (Hammes and Kovachi, 1962; Eigen, 1960).

SUMMARY

Stability constants of $MgATP^{2-}$ and $MgADP^{-}$ have been obtained by measuring the spectral shifts of the Mg-8-hydroxyquinoline complex in the presence and absence of nucleotide. Reasonable confirmation of the value for the $MgATP^{2-}$ complex has been obtained from pH-titration measurements. For this latter technique, a programme, suitable for analysis by a 1620 IBM Computer, has been written.

Estimates of the stability constants of $CaATP^{2-}$ by pH-titration, $CaADP^{-}$ by spectrophotometry and $MnADP^{-}$ by spectrophotometry and electron paramagnetic resonance techniques, have also been obtained.

CHAPTER III

THE METAL ACTIVATION AND INHIBITION OF THE REACTION CATALYSED BY ATP:CREATINE PHOSPHOTRANSFERASE

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THE METAL ACTIVATION AND INHIBITION OF THE REACTION CATALYSED BY ATP:CREATINE PHOSPHOTRANSFERASE

INTRODUCTION

Many enzymes, which require the addition of metal ions for maximum activity, show some residual activity in the absence of added metal ions. However, there has been little investigation of the reasons for this activity though it appears likely that it is due to the presence of trace amounts of activating metal ions in the reaction mixtures. Thus, the presence of sufficient amounts of activating metal ions to account for the observed residual activity was demonstrated by Kachmar and Boyer (1953) for pyruvate kinase and by Malmström (1955a) for phospho-pyruvate hydratase.

Alternatively, any residual activity due to the presence of activating metals should be eliminated by the use of suitable chelating agents. Portmann (1957) and Milstein (1961a) showed that the residual activity observed with preparations of alkaline phosphatase and phosphoglucomutase respectively, could be suppressed with EDTA, indicating that the observed activity was probably due to traces of metals.

If kinetic experiments, using low concentrations of

added metal ions, are to be carried out with an enzyme, then it is desirable that the activity in the absence of added metal ion should be negligible. This chapter describes an investigation into the residual activity of creatine kinase and the effect of chelating agents and a number of metal ions on the activity.

MATERIALS AND METHODS

N-Ethylmorpholine, purified as described in Chapter II, was adjusted to pH 8.0 with 5 N HCl.

ADP and PC were prepared as described in Chapter II.

All metal salts, AnalaR, were made up in stock solution as the chloride (Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Sr^{2+}, Cd^{2+}, Cu^{2+}), acetate (Ba^{2+}, Zn^{2+}), sulphate (Be^{2+}, Ni^{2+}, Cr^{3+}, VO^{2+}) or nitrate (UO_2^{2+}).

Imidazole was purchased from Eastman Organic Chemicals, histidine (HCl) from the California Corporation for Biochemical Research and cysteine (HCl) from the Sigma Chemical Co.

Crystalline creatine kinase was prepared from frozen rabbit muscle by the method of Kuby, Noda and Lardy (1954a). After recrystallisation, the crystals were collected by centrifugation at 0°, dissolved in 10^{-3} M N-ethylmorpholine (pH 8.0) containing 10^{-3} M EDTA and dialysed against three changes (250 vol.) of the same solution over a period of three days. The EDTA was removed by dialysing against

three changes (250 vol.) of N-ethylmorpholine (pH 8.0) for the same time. The stock enzyme solution, containing 30 mg. of protein per ml., was stored at 0°, no loss of activity being observed after two years. The crystalline enzyme was also stable for the same time period, when stored as a suspension at -10° in 56% (v/v) ethanol.

Protein was estimated colorimetrically by the biuret method of Gornall, Bardawill and David (1949), using crystalline bovine albumin as the standard.

Creatine was estimated by a slight modification of the method described by Rosenberg, Ennor and Morrison (1956) for the estimation of arginine. To 1.0 ml. of reaction mixture was added 0.3 ml. of 3 N NaOH (containing 0.04 M EDTA), 1.0 ml. of α-naphthol-diacetyl reagent (12.5 g. of α-naphthol and 6.25 ml. of diacetyl in 250 ml. n-propanol) and the volume was made to 3.0 ml. Scans were carried out, with standard solutions of creatine, on a Beckman DK2 ratio-recording spectrophotometer from 400 mμ to 600 mμ in 1 min. These showed that the coloured complex formed had an absorption maximum at 535 mμ and that the extinction was maximal after a time interval of 10 min. at 27°. Under these conditions 0.04 μmole of creatine gave an extinction of 0.280 when measured in a cell of 1 cm. light path using a Shimadzu (QR-50) spectrophotometer.

Unless otherwise stated, the following procedure was used to determine enzymic activity. To 3 ml. graduated tubes was added 0.2 ml. of N-ethylmorpholine buffer (0.5 M, pH 8.0), 0.1 ml. of PC (0.1 M) and ADP and $MgCl_2$ in a total volume of 1.0 ml. After the addition of the reagents, the tubes were stored in an ice-bath and, before the addition of enzyme (0.02 ml., equivalent to 0.9 µg. of protein), were equilibrated at 30° for 3 min. At the end of the incubation period (1-2 min.), the reaction was stopped by the addition of 0.3 ml. of 3 N NaOH (containing 0.04 M EDTA) and the creatine released from PC estimated as described above. When the stock enzyme solution was diluted 1 in 1000 with N-ethyl-morpholine buffer (10^{-3} M, pH 8.0), the enzyme was stable for at least 2 hr. at 0°; greater dilutions resulted in a rapid loss of enzyme activity.

The heavy metal content of PC was reduced by passing a solution (5 g. in 100 ml.) through a column (1.5 x 10 cm.) of Dowex-50 (Na^+), 200-400 mesh, and recrystallising the PC in the effluent solution from 80% ethanol. ADP (10^{-2} M) was treated similarly except that the ADP was not recrystallised.

The analyses for Mg^{2+}, Ca^{2+} and Mn^{2+} were carried out by Mr. J. David, Division of Plant Industry, C.S.I.R.O., Canberra, using a Uvispek atomic absorption spectrometer.

RESULTS

The activating metal content of the reaction components and the effect of resin treatment of the substrates on the residual activity of the enzyme.

In preliminary experiments, the creatine kinase preparation was found to have a residual activity of 17% with ADP (2.8×10^{-4} M) and PC (10^{-2} M) as compared with the activity found in the presence of added Mg^{2+} (2×10^{-4} M). As this could be due to contamination of one of the reaction components with one of the activating ions, Mg^{2+}, Ca^{2+} or Mn^{2+}, spectrographic analyses were carried out. The results (Table 7) indicate that only ADP and PC made significant contributions to the concentrations of activating metal ions. Values for only Mg^{2+} and Ca^{2+} are reported, as Mn^{2+} was not detected in any of the constituents. Table 7 also shows the effect of treating solutions of ADP and PC with Dowex-50 (Na^{+}). The concentrations of Mg^{2+} and Ca^{2+} were considerably reduced, with the result that the residual activity was halved. Thus, under the experimental conditions, the activity in the absence of Mg^{2+} was not greater than 4% of the maximum attainable in the presence of saturating amounts of both Mg^{2+} and ADP^{3-}. Divalent metal ions would have been much more effectively removed by treatment with a chelating resin, but such a resin was

TABLE 7

The Mg^{2+} and Ca^{2+} contents of reaction mixtures containing ADP and PC based on spectrographic analysis

The values quoted are the final concentrations of Mg^{2+} and Ca^{2+} in the reaction mixture due to the addition of ADP (2.8×10^{-4} M), PC (10^{-2} M), N-ethylmorpholine (0.1 M) and creatine kinase (0.9 µg.) in a total volume of 1 ml. The residual activity is expressed as a percentage of that obtained with added Mg^{2+} (2×10^{-4} M).

Before Dowex-50 treatment

Compound added	Mg^{2+} ($M \times 10^6$)	Ca^{2+} ($M \times 10^6$)
ADP	0.5	2
PC	2.8	18
N-ethylmorpholine	0.1	0.4
Creatine kinase	0.0003	0.0007

Total metal ion = 2.4×10^{-5} M

Residual activity = 17%

After Dowex-50 treatment

ADP	0.2	0.5
PC	0.8	2.6

Total metal ion = 4×10^{-6} M

Residual activity = 8%

(N.B. 0.9 µg. creatine kinase/ml. represents an approximately 10^{-8} M solution. This contains approximately 10^{-9} M of $Ca^{2+} + Mg^{2+}$.)

not available at the time these experiments were carried out.

It is interesting to note that the creatine kinase preparation still contained about 0.1 µmole of divalent metal ion (Mg^{2+} + Ca^{2+}) per µmole of protein, even after dialysis against 10^{-3} M EDTA for three days.

Effect of chelating agents on the activity of creatine kinase.

EDTA, at a concentration of 5 x 10^{-5} M or above, was found to cause complete inhibition of the residual activity when tested with substrate solutions which had not been heated with Dowex-50 (Na^{+}). This concentration of EDTA was slightly higher than the total concentration of activating metal ion added as contaminants by ADP and PC (Table 7).

Lower concentrations of EDTA caused almost a two-fold increase in the residual enzymic activity (Fig. 7). This activating effect of low concentrations of EDTA was also observed in the presence of added Mg^{2+}, suggesting that the enzymic activity was limited by virtue of the presence of heavy metal ions in the reaction mixtures.

Cysteine and imidazole, in the concentration range from 4 x 10^{-5} M to 10^{-3} M, were found to have no effect on the activity of creatine kinase, either in the presence or absence of Mg^{2+}. Histidine showed an activating effect,

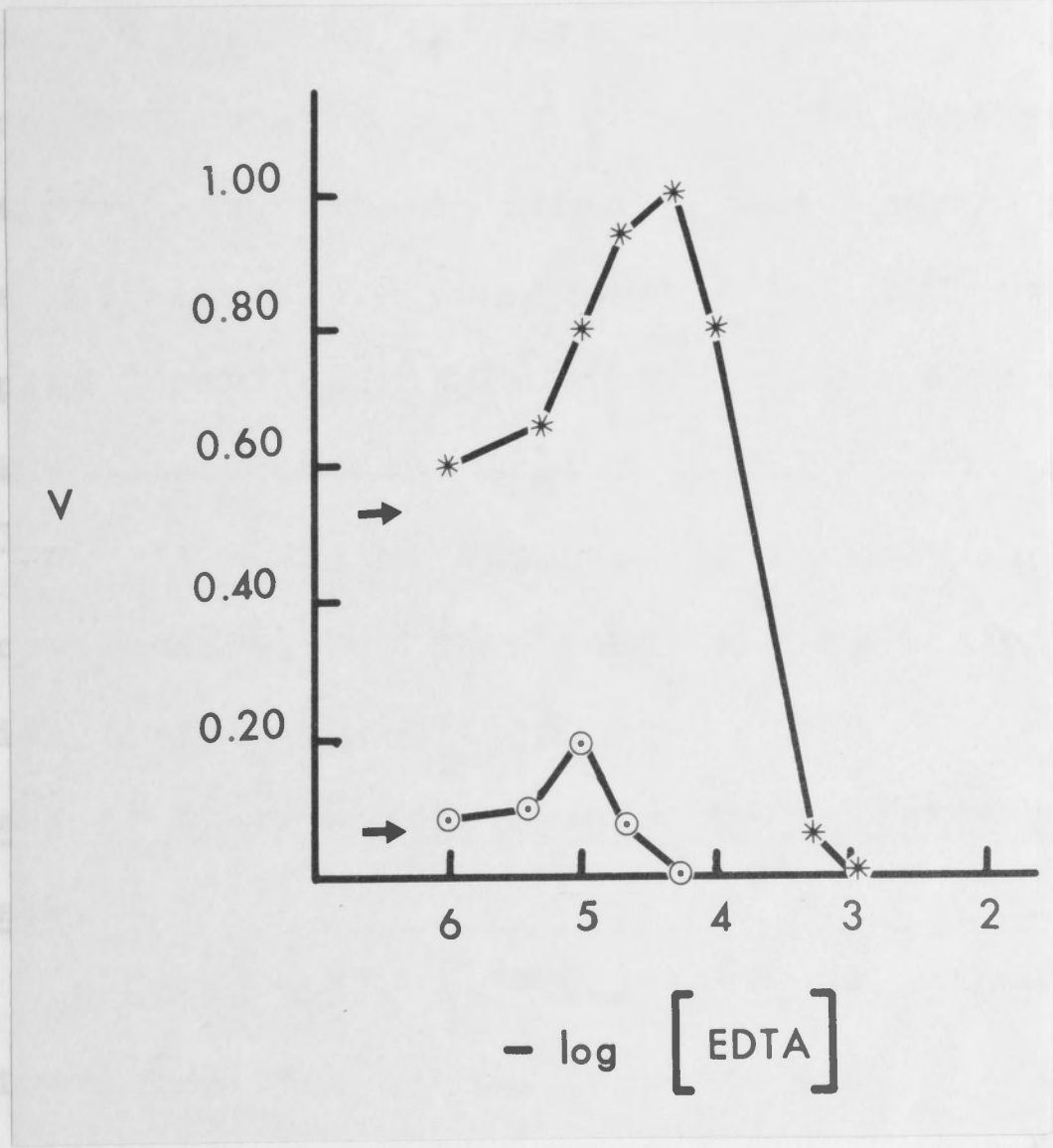

Fig. 7. The effect of EDTA on the reaction catalysed by creatine kinase. The reaction mixture contained N-ethylmorpholine (pH 8.0), 0.1 M; ADP, 2.8×10^{-4} M; PC, 10^{-2} M and creatine kinase (0.9 µg.). Total volume 1.0 ml., temp., 30°. The velocity is expressed as optical density measured at 535 mµ. ✳ — ✳ , $MgCl_2$, 5×10^{-4} M; ⊙ — ⊙ , no added metal ion. The position of the arrows indicates the observed velocity in the absence of EDTA.

the activation increasing with increasing histidine concentration. No inhibition was observed up to a concentration of 5×10^{-3} M. Thus it appears that histidine activates by removing heavy metal ions but does not reduce the concentration of Mg^{2+} or Ca^{2+} by complex formation. 8-Hydroxyquinoline also activated in the concentration range from 0.75×10^{-4} M to 0.3×10^{-3} M. It would be expected to inhibit at higher concentrations, but this could not be tested because of its limited solubility.

Effect of other metal ions on the activity of creatine kinase.

Besides Mg^{2+}, Ca^{2+} and Mn^{2+}, which were known to activate creatine kinase (Kuby et al., 1954b), the enzyme was also found to be activated by Co^{2+}. Slight inhibitory effects were observed with the other alkaline earth ions, Sr^{2+}, Ba^{2+} and Be^{2+}, while stronger inhibition was observed with Ni^{2+} and Cr^{3+}. These effects were reversible. Complete and irreversible inhibition was obtained with Cd^{2+}, VO^{2+}, Zn^{2+}, Cu^{2+} and UO_2^{2+}.

A time-dependent denaturation of the diluted enzyme.

As was noted in the Materials section of Chapter II, all PC preparations contained a small amount of free creatine and a blank determination was carried out with each set of enzyme assays. This blank value could also

be obtained by determining the activity at different time intervals and extrapolating back to zero time. With experiments using high concentrations of magnesium (see Chapter V) it was found that the value of the extrapolated blank was much higher than the measured value. This is illustrated by the starred points in Fig. 8. The extrapolated blank corresponded to an optical density of 0.100 at 535 mμ, while the measured blank was only 0.040. When the activity was determined at shorter time intervals (open circles in Fig. 8) a value of the extrapolated blank identical to the directly determined value was obtained. The biphasic nature of the reaction is consistent with a time-dependent denaturation of the enzyme, which could be due to the presence of heavy metal ions, introduced as trace contaminants of the reaction components.

When the same experiment was carried out in the presence of 10^{-5} M EDTA, which concentration would be too low to have any appreciable effect on the magnesium concentration, a linear reaction was observed up to $1\frac{1}{2}$ min. (full circles in Fig. 8). This result suggests the removal of trace amounts of heavy metal ions. The use of EDTA at this concentration was adopted for all experiments where its effect on the free Mg^{2+} concentration could be considered to be negligible. It

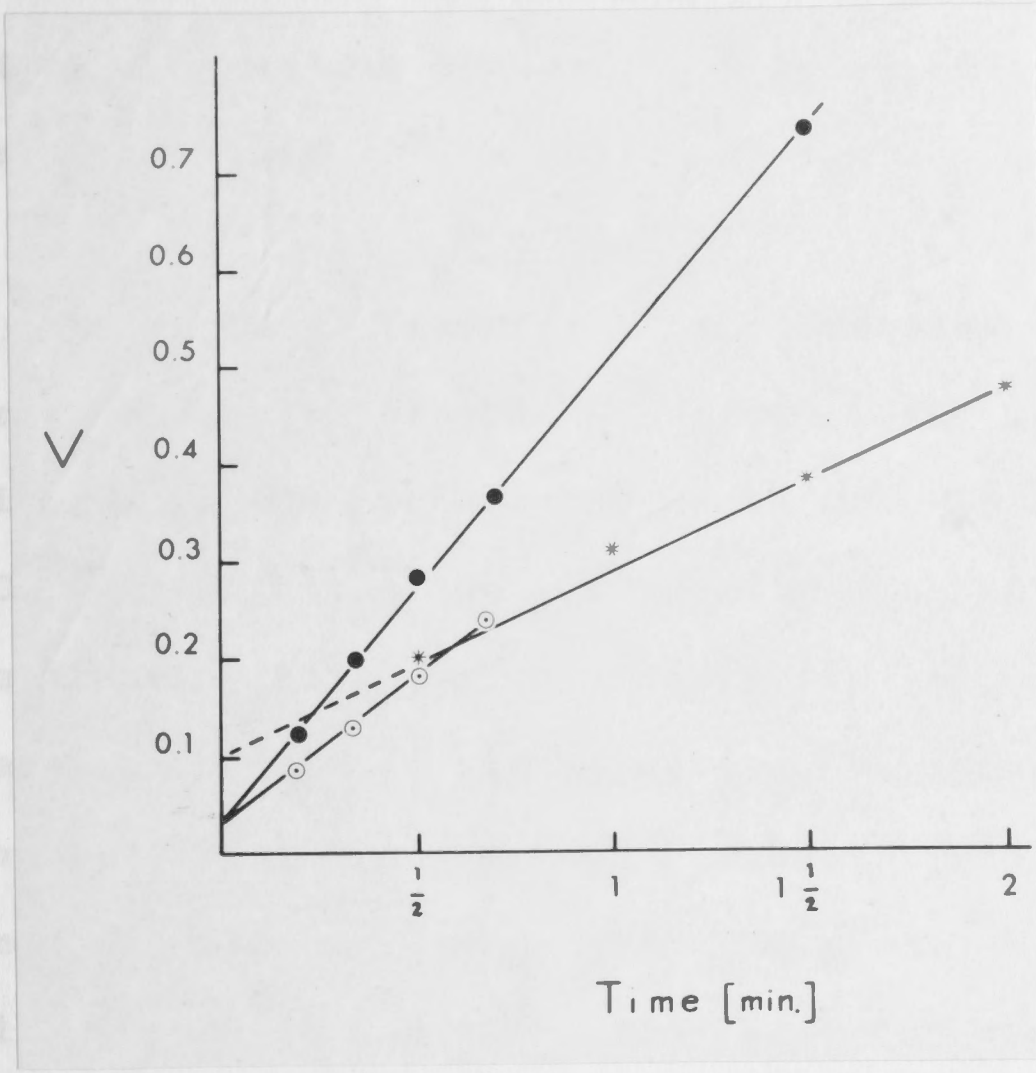

Fig. 8. The apparent biphasic nature of the reaction catalysed by creatine kinase. The reaction mixture contained N-ethylmorpholine (pH 8.0), 0.1 M; PC, 10^{-2} M; ADP, 2.4×10^{-4} M; $MgCl_2$, 3.1×10^{-4} M; and creatine kinase (0.9 µg.). Total volume, 1.0 ml., temp., 30°. Velocity is expressed as extinction measured at 535 mµ. ⊙—⊙, and ✳—✳, no added EDTA; ●—●, EDTA, 10^{-5} M.

was also found that the diluted enzyme was more stable in the presence of low concentrations of EDTA and for the results described in Chapters V and VI, the stock solution of creatine kinase was diluted, 1 in 1000 in buffer containing 10^{-5} M EDTA.

DISCUSSION

The residual activity of the creatine kinase preparation in the absence of added metal ion could be attributed to the presence of small amounts of Mg^{2+} and Ca^{2+} which are added principally with ADP and PC. The activating effect of low concentrations of EDTA in particular, and of histidine and 8-hydroxyquinoline, is consistent with the removal of heavy metal ions. No attempt was made to demonstrate the presence of heavy metals, though it was shown that creatine kinase is susceptible to a large number of these. This susceptibility is in accord with the demonstrations by Rosenberg and Ennor (1955) and by Mahowald, Noltmann and Kuby (1962), that free -SH groups on the enzyme were essential for catalytic activity. A time-dependent denaturation of the enzyme was also attributed to the presence of trace amounts of heavy metals, as the effect was eliminated by 10^{-5} M EDTA.

Since higher concentrations of EDTA inhibited the reaction, almost certainly due to the removal of the

activating metal ion, it would be expected that 8-hydroxyquinoline would have a similar effect. However, this compound is not very soluble and its effect at high concentrations could not be tested. Histidine was found to have an activating effect, the activity increasing almost as a linear function of the logarithm of its concentration, but it apparently did not combine sufficiently strongly with the activating metals to cause any effective inhibition.

These results are essentially similar to those observed by Milstein (1961b) for the activation of phosphoglucomutase by chelating agents. This effect was shown to be due to the removal of heavy metal contaminants. Milstein also found that cysteine activated phosphoglucomutase. However, no effect on creatine kinase was detected at the concentrations of cysteine used in this work.

These findings on the activation and inhibition of creatine kinase confirm its absolute requirement for a divalent metal ion. Thus, the enzyme conforms to the essential properties elaborated by Malmström and Rosenberg (1959) for classification as a metal-enzyme.

SUMMARY

The observed residual activity of the creatine kinase preparation could be satisfactorily explained by

the presence of sufficient amounts of activating divalent metal ions in the components of the reaction mixtures. Low concentrations of EDTA were found to have both an enhancing effect on the enzymic activity and a stabilising effect on the enzyme, which effects are probably due to the removal of heavy metal ions.

CHAPTER IV

KINETIC STUDIES OF THE REVERSE REACTION

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INTRODUCTION

The first detailed study of the reaction catalysed by creatine kinase, which used a crystalline preparation of the enzyme, was carried out by Kuby, Noda and Lardy (1954b). They found that for the forward direction, maximal velocity was obtained with a 1:1 ratio of Mg:ATP; whereas for the reverse direction a somewhat higher ratio of metal to ADP was required. These data were taken to indicate that the metal complexes of ATP and ADP were the active substrates of the enzyme. Such conclusions were consistent with the fact that the species $MgATP^{2-}$ and $MgADP^{-}$ were known to be present in solution though the estimates of the values available at the time for the stability constants of these complexes were much too low to be in strict accordance with the experimental findings, i.e., the value of 830 M^{-1} available for $MgATP^{2-}$, which was that reported by Burton and Krebs (1953), would not have given sufficient $MgATP^{2-}$ present in solution to account for the fact that maximum velocity was observed at an Mg:ATP ratio of 1:1 (cf. this thesis; Chapter I). The discrepancy would have been even greater with $MgADP^{-}$

particularly as the reaction was carried out at pH 7.0, this being only slightly above the pK_a for the terminal phosphate group of ADP, and it could have been estimated from Burton and Kreb's (1953) value of 360 M^{-1} that appreciable concentrations of the four species, ADP^{3-}, $MgADP^{-}$, $HADP^{2-}$ and MgHADP would have been present.

Experiments carried out by Kuby et al. (1954b) also showed that ADP inhibited the forward reaction, competitively with respect to ATP and non-competitively with respect to creatine. These findings were interpreted in terms of two active sites in juxtaposition on the enzyme molecule, one for the magnesium-nucleotide complexes and the other for creatine or PC. It was noted that excess of Mg^{2+} over nucleotide inhibited the reaction in both directions, but the possibility of an Mg-enzyme complex being an intermediate in the enzymic reaction was not considered.

Further kinetic studies have been carried out in the forward direction by Noda, Nihei and Morales (1960) and in the reverse direction by Nihei, Noda and Morales (1961). In these studies it was assumed that the metal-nucleotide complex was the "true" substrate of the reaction in each case. By assigning values of 90,000 M^{-1} and 2,000 M^{-1} to the values for the stability constants

of $MgATP^{2-}$ and $MgADP^{-}$ respectively, these workers concluded that this simple assumption was valid, with the qualification that, for the reverse reaction, it was necessary to consider the interaction of ADP^{3-} with the enzyme, presumably as a competitor for the same site as $MgADP^{-}$.

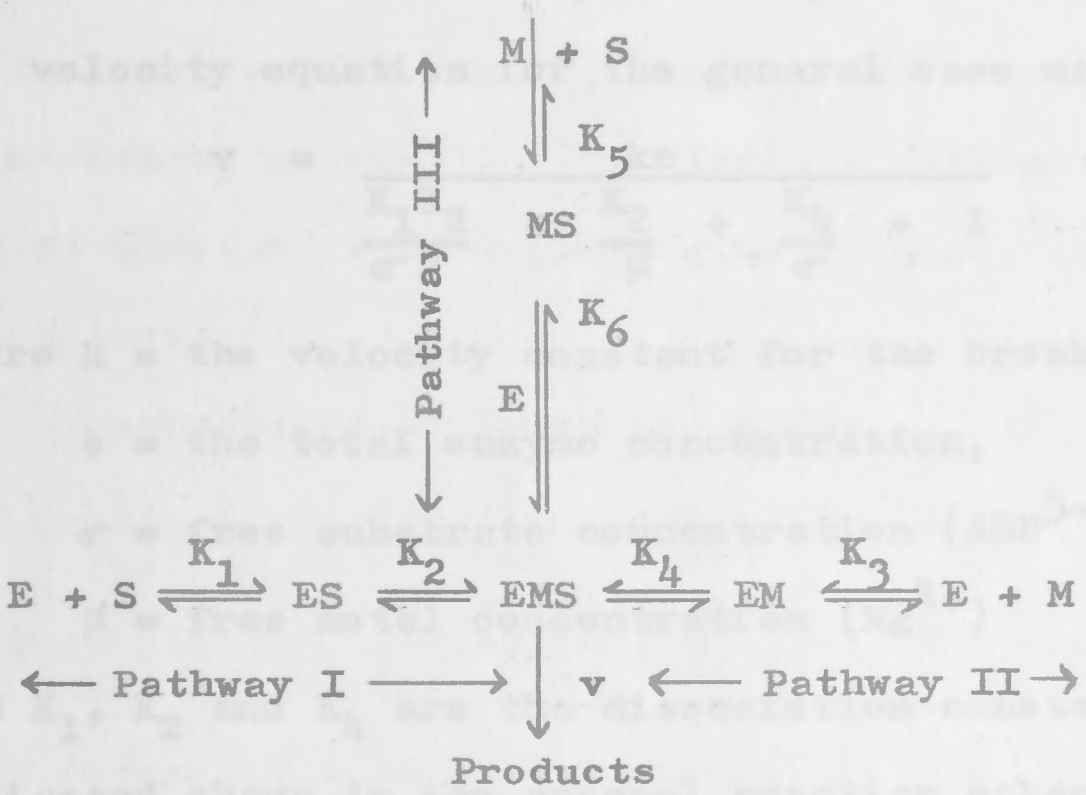
In none of these previous studies was the kinetic analysis extended to consider the possible interaction of other species (viz., Mg^{2+}, ADP^{3-}, ATP^{4-}) with the enzyme as being steps along the reaction pathway. This chapter describes initial kinetic experiments carried out in the reverse reaction catalysed by creatine kinase, using a more general approach, by which the independent interaction of the three species, Mg^{2+}, ADP^{3-} and $MgADP^{-}$ with the enzyme, was considered. It is shown that the results obtained were consistent with all of the three species being operative in the formation of an EMS complex.

THEORY

The conversion of PC and ADP to creatine and ATP respectively, which takes place in the presence of ATP:creatine phosphotransferase and Mg^{2+}, was assumed to occur via the formation of an active enzyme-Mg-ADP-PC complex. Furthermore, it was considered that this

complex is formed by three sets of bimolecular reactions which take place in a number of parallel sequences. In the first instance, the concentration of PC was held constant and Mg^{2+} activation was studied in relation to ADP^{3-}. Thus it was supposed that the velocity of the reaction was a function of the concentration of the enzyme-Mg-ADP complex and that the concentration of PC altered the velocity only by a constant factor.

In considering the interaction of the enzyme with free metal ion, free substrate and the metal substrate complex according to the following binary reactions



and in deriving the velocity equation, a number of assumptions, in addition to those stated above, have been made. It has been assumed that: (1) all reactions leading to the formation of EMS are in rapid equilibrium so that Michaelis-Menten, rather than Briggs-Haldane,

kinetics may be applied; (2) the same complex is formed irrespective of the pathway of formation (the exact nature of the complex need not be specified); (3) there is no competitive inhibition between the various species for sites on the enzyme surface; (4) the amounts of EM, ES and EMS formed are not sufficient to affect appreciably the concentrations of M, S or MS.

Under equilibrium conditions, the concentration of EMS will be independent of the pathway by which it is formed so that

$$K_1K_2 = K_3K_4 = K_5K_6 \qquad (1)$$

The velocity equation for the general case may be written

$$v = \frac{ke}{\frac{K_1K_2}{\sigma\mu} + \frac{K_2}{\mu} + \frac{K_4}{\sigma} + 1} \qquad (2)$$

where k = the velocity constant for the breakdown of EMS,

e = the total enzyme concentration,

σ = free substrate concentration (ADP^{3-}),

μ = free metal concentration (Mg^{2+})

and K_1, K_2 and K_4 are the dissociation constants as indicated above in the general reaction scheme. Equation (2) can, of course, be written in terms of other sets of constants, in accordance with equation (1).

Equation (2) may be arranged in reciprocal form to give

$$\frac{1}{v} = \frac{1}{ke} \cdot \frac{1}{\sigma}\left\{\frac{K_1K_2}{\mu} + K_4\right\} + \frac{1}{ke}\left\{\frac{K_2}{\mu} + 1\right\} \quad (3)$$

so that a plot of $\frac{1}{v}$ against $\frac{1}{\sigma}$ should be linear, where μ is held constant at μ', with intercepts at $\frac{1}{V_{\mu'}}$ and $-\frac{1}{K_{\mu'}}$, where these are defined as

$$V_{\mu'} = \frac{ke}{\frac{K_2}{\mu'} + 1} \quad (4)$$

and

$$K_{\mu'} = \frac{\frac{K_1K_2}{\mu'} + K_4}{\frac{K_2}{\mu'} + 1} \quad (5)$$

In this way values for $K_{\mu'}$ and $V_{\mu'}$ for various values of μ' can be obtained. (Similarly plotting $1/v$ against $1/\mu$ at fixed σ' values would give a series of $K_{\sigma'}$ and $V_{\sigma'}$ values).

Rearranging equation (4) gives

$$\frac{1}{V_{\mu'}} = \frac{1}{\mu'} \cdot \frac{K_2}{ke} + \frac{1}{ke} \quad (6)$$

so that plotting $\frac{1}{V_{\mu'}}$ against $\frac{1}{\mu'}$ gives a straight line with intercept $-\frac{1}{K_2}$ on the abscissa.

Rearranging equation (5) gives

$$K_{\mu'}\left\{\frac{K_2}{\mu'} + 1\right\} = \frac{K_1K_2}{\mu'} + K_4 \quad (7)$$

so that plotting $K_{\mu'}\left\{\frac{K_2}{\mu'} + 1\right\}$ against $\frac{1}{\mu'}$ gives

intercepts K_4 and $-\frac{K_4}{K_1K_2}$ on the ordinate and abscissa respectively.

Then by using equation (1) values may be assigned to K_1 and K_3 and as K_5 is determined independently, to K_6.

The concentration of total Mg and total ADP required to maintain μ at a fixed value while σ is varied was calculated from $S = \frac{K_5M}{\mu} - K_5 + M - \mu$ (8)

where S = total ADP and M = total Mg.

This was derived from the equation

$$K_5 = \frac{\sigma . \mu}{z}$$

as $\sigma = S - z$ and $\mu = M - z$ where $z = MgADP^-$.

The same velocity equation applies when the reaction of PC and Mg^{2+} with the enzyme is considered, except that K_1 and K_4 now represent the dissociation constants for the interaction of PC with the enzyme and enzyme-metal complex respectively. The values for the four possible dissociation constants can be obtained in an analogous fashion to that described above.

MATERIALS AND METHODS

Materials were as described previously in Chapters II and III, PC and ADP having been subjected to the resin treatment described in Chapter III.

Creatine was estimated as described in Chapter III.

The same procedure was adopted for the measurement of enzymic activity, the creatine release being estimated at two time intervals to ensure that initial velocities were being measured.

RESULTS

The pH optima of the forward and reverse reaction catalysed by creatine kinase have been reported to be at about pH 9.0 and pH 7.0 respectively (Kuby et al., 1954b). Most of the studies reported in this chapter were carried out at pH 8.0 so that values obtained for the dissociation constants of the intermediate complexes could be correlated with the values reported in Chapter VII for the forward reaction. At pH 8.0, two values were used for the stability constant of $MgADP^-$; 1,000 M^{-1} as determined by Smith and Alberty (1956) and 4,150 M^{-1} as reported in Chapter II. Thus, any errors introduced by uncertainty as to the magnitude of this value should have been lessened. Some studies were also carried out at pH 7.0 using the value of 2,000 M^{-1} at this pH as reported in Chapter II. N-Ethylmorpholine-HCl was used to maintain the pH and ionic strength of the reaction solutions. As was demonstrated in Chapter II, the interaction of this buffer with Mg^{2+} is negligible.

Activation of creatine kinase in relation to ADP^{3-} at pH 8.0.

The results, using K_5 = 1 mM (stability constant =

1,000 M^{-1}) (Fig. 9) showed that reasonably good straight lines are obtained when 1/v is plotted against $1/ADP^{3-}$ $(1/\sigma)$ at various fixed values of free Mg^{2+} (μ') according to equation (3). The values for $K_{\mu'}$ and $V_{\mu'}$, as calculated by a statistical weighted mean squares method (Wilkinson, 1961), are listed in Table 8. These values were used in drawing the lines in Fig. 9. The fact that the lines extrapolated back to give an average value for $K_{\mu'}$ of 1.0×10^{-4} M indicated that the concentration of Mg^{2+} had little or no effect on the binding of ADP^{3-}. The plots shown in Figs. 10 and 11, corresponding to equations (6) and (7) respectively, show that straight lines are obtained when $1/\mu'$ is plotted against $1/V_{\mu'}$ (Fig. 10) and $K_{\mu'}$ $(\frac{K_2}{\mu'} + 1)$ (Fig.11). For the latter plot an average value of $K_{\mu'}$ was used. Fig. 10 yielded a value for K_2 while Fig. 11 gave values for K_4 and $\frac{K_4}{K_1K_2}$.

From the relationship shown in equation (1), the remaining values for K_1, K_3 and K_6 were calculated. A summary of these values is given in Table 9.

Similar studies were carried out using K_5 = 0.24 mM (stability constant = 4,150 M^{-1}). The results from two sets of experiments are shown in Table 10 and the plots for the first set in Fig. 12. It is seen that there is

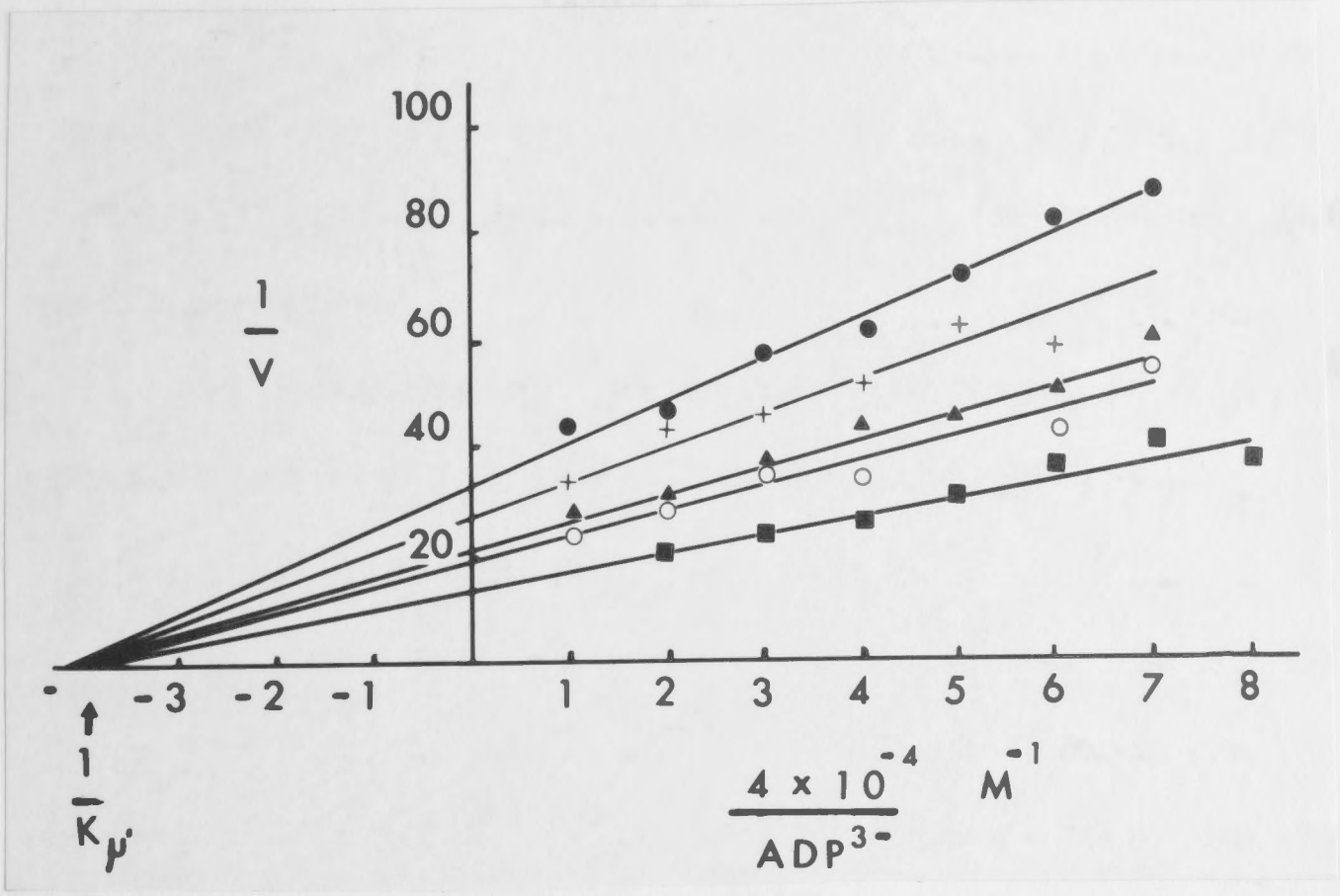


Fig. 9. Effect of the concentration of ADP^{3-} on the initial velocity of the reaction at various fixed concentrations of Mg^{2+}. The concentrations of ADP^{3-} and Mg^{2+} were adjusted by varying the total concentrations of ADP and Mg, with a value of 1,000 M^{-1} for the stability constant of $MgADP^{-}$. The reaction mixtures contained N-ethylmorpholine (pH 8.0), 0.1M; PC (10^{-2} M) and creatine kinase, 0.9 μg., as well as the indicated amounts of Mg^{2+} and ADP^{3-}. Total volume, 1.0 ml., temp. 30°. Velocity is expressed as μmoles of creatine/μg. of creatine kinase/min. ■—■ Mg^{2+}, 4 x 10^{-4} M; ○—○ Mg^{2+}, 2 x 10^{-4} M; ▲—▲ Mg^{2+}, 1.33 x 10^{-4} M; +—+ Mg^{2+}, 1 x 10^{-4} M; ●—● Mg^{2+}, 0.66 x 10^{-4} M.

TABLE 8

Summary of the values obtained for $K_{\mu'}$ and $V_{\mu'}$ at various fixed concentrations of Mg^{2+} (μ'), at pH 8.0 with K_5 = 1 mM.

All K values are expressed in terms of M x 10^4, while those for $V_{\mu'}$ are given in arbitrary units.

Mg^{2+} Concentration (μ')	$K_{\mu'}$	$V_{\mu'}$
4.0	1.1	0.72
2.0	1.0	0.50
1.33	1.0	0.45
1.0	0.9	0.34
0.66	1.0	0.29

Average = 1.0

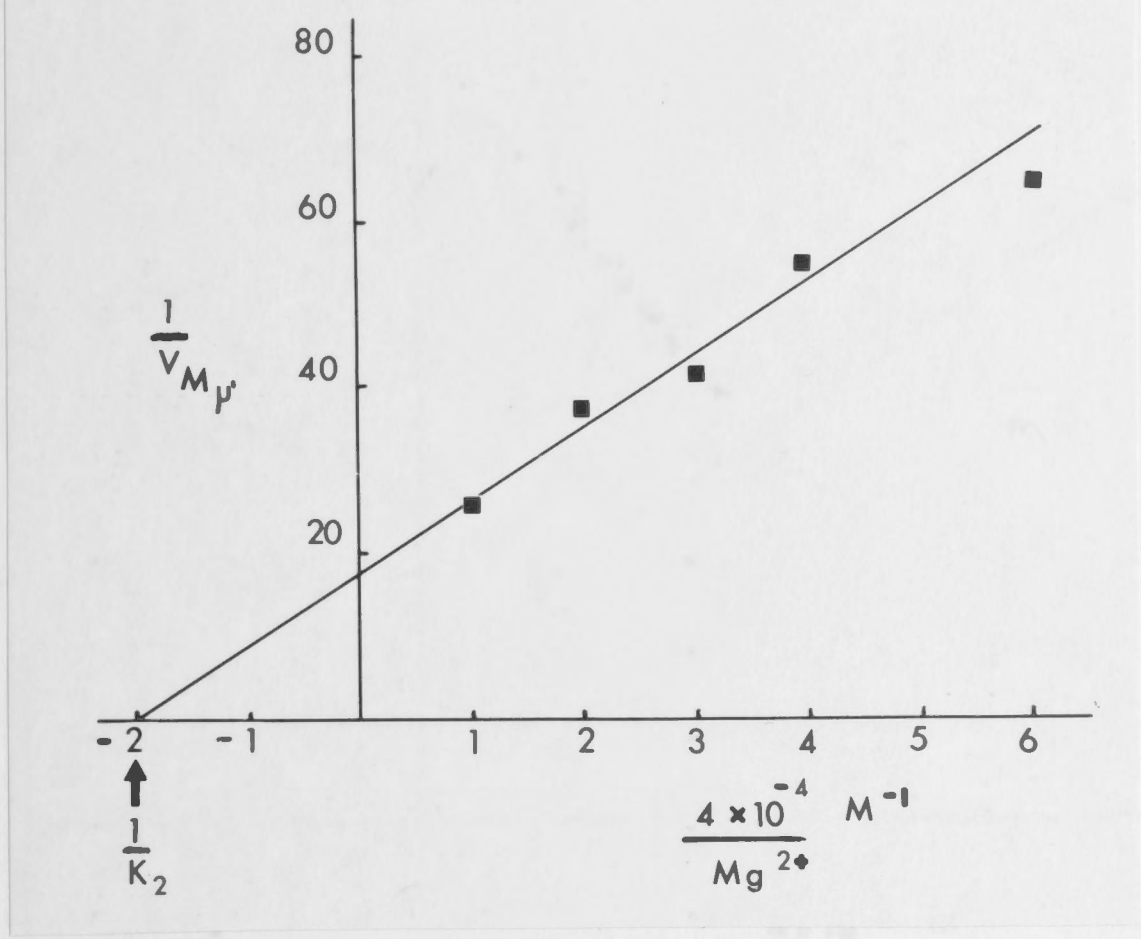


Fig. 10. Plot of the reciprocals of the maximum velocities obtained in the presence of fixed amounts of Mg^{2+} and saturating amounts of ADP^{3-} against the reciprocals of the concentrations of Mg^{2+} (equation 6). Maximum velocity values were obtained from the plot shown in Fig. 9 and are expressed as μmoles of creatine/μg. of creatine kinase/min.

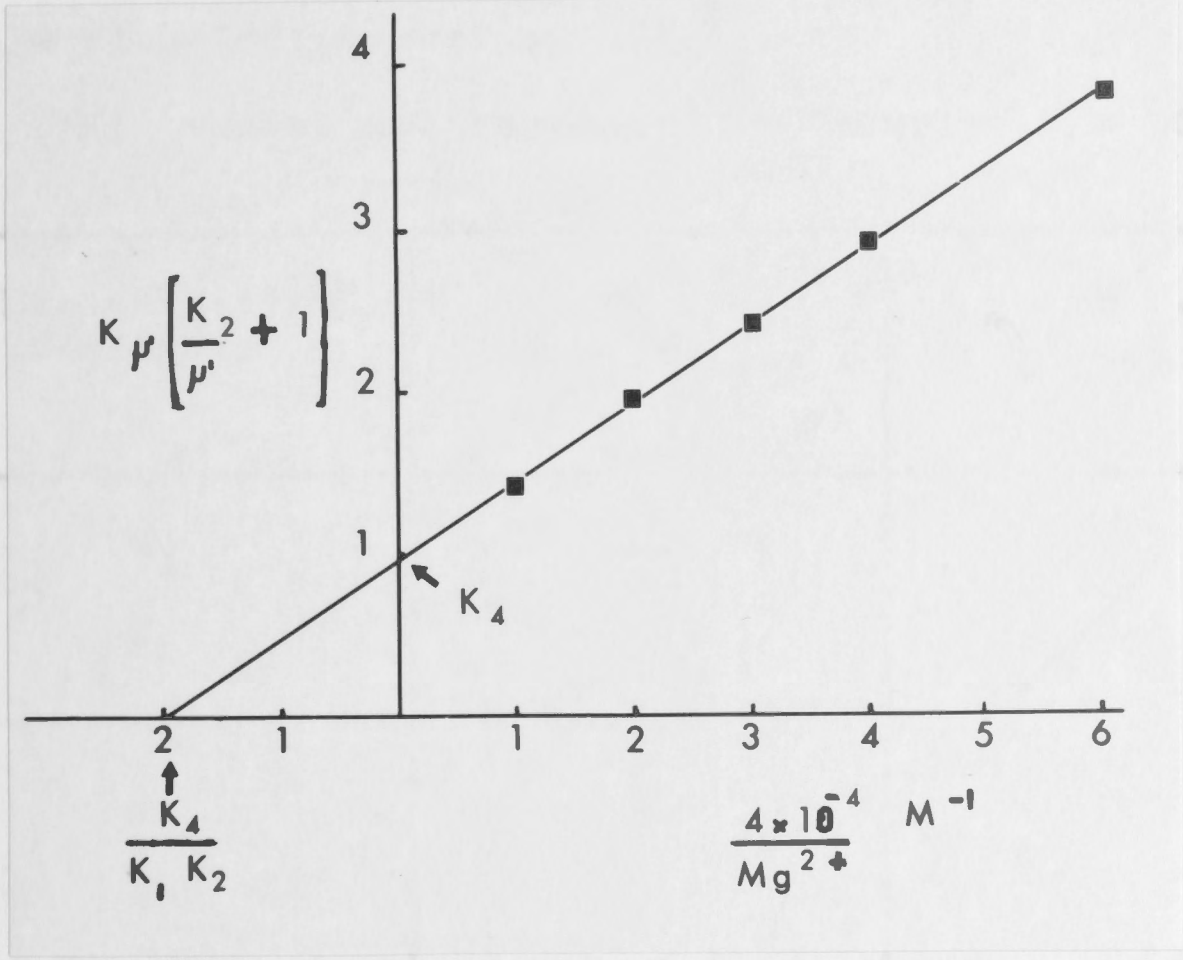


Fig. 11. Plot of the calculated values for $K_\mu \left\{\frac{K_2}{\mu'} + 1\right\}$ against the reciprocals of the concentrations of Mg^{2+} (equation 7).

TABLE 9

Summary of the dissociation constants for the various reactions leading to the formation of the active enzyme-metal-substrate complex. The values for the constants at pH 7.0 have been corrected to allow for the nucleotide present as $HADP^{2-}$.

All values are expressed in terms of M x 10^4.

Dissociation constants	pH 8.0 $K_5 = 10$	pH 7.0 $K_5 = 5$
K_1	1.0	0.7
K_2	2.1	3.2
K_3	2.1	3.2
K_4	1.0	0.7
K_6	0.2	0.4

TABLE 10

Summary of the values obtained for $K_{\mu'}$ and $V_{\mu'}$ at various fixed concentrations of Mg^{2+} (μ'), at pH 8.0 with K_5 = 0.24 mM.

All K values are expressed in terms of M x 10^4, while those for $V_{\mu'}$ are given in arbitrary units.

Mg^{2+}	$K_{\mu'}$	$V_{\mu'}$
4.0	1.1 (0.8)*	0.81
2.0	1.3 (1.6)	0.58
1.33	1.5 (1.2)	0.54
1.0	1.7 (1.8)	0.40
0.66	1.9 (1.4)	0.33

Average = 1.5 (1.4)

*Figures in parenthesis represent the values obtained for $K_{\mu'}$ from a second set of experiments.

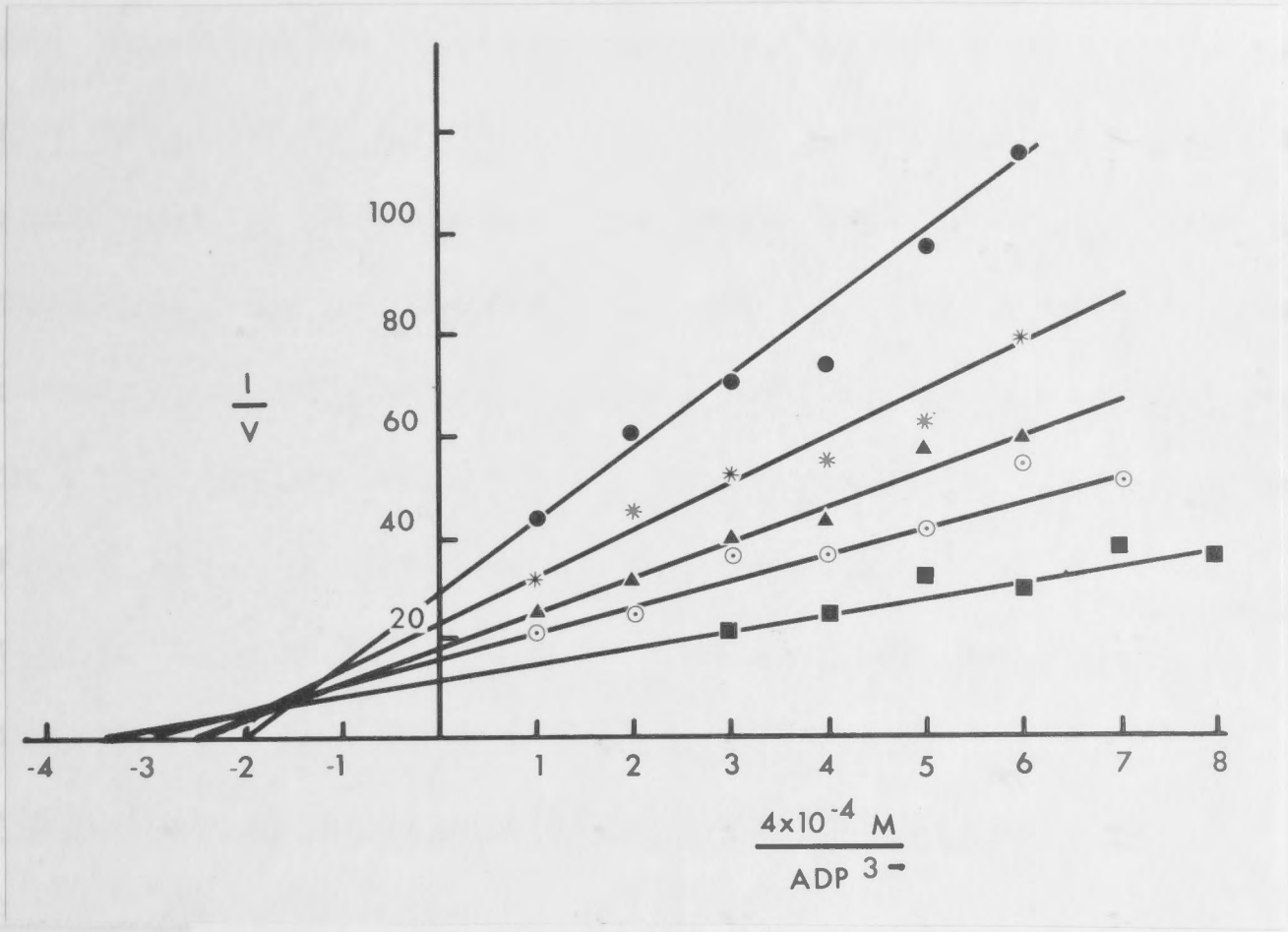


Fig. 12. Effect of the concentration of ADP^{3-} on the initial velocity of the reaction at various fixed concentrations of Mg^{2+}. The concentrations of ADP^{3-} and Mg^{2+} were adjusted by varying the total concentrations of ADP and Mg, with a value of 4,150 M^{-1} for the stability constant of $MgADP^{-}$. The reaction mixtures contained N-ethylmorpholine (pH 8.0), 0.1 M; PC (10^{-2} M) and creatine kinase, 0.9 µg., as well as the indicated amounts of Mg^{2+} and ADP^{3-}. Total volume, 1.0 ml., temp. 30°. Velocity is expressed as µmoles of creatine/µg. of creatine kinase/min. ■—■ Mg^{2+}, 4 x 10^{-4} M; ⊙—⊙ Mg^{2+}, 2 x 10^{-4} M; ▲—▲ Mg^{2+}, 1.33 x 10^{-4} M; ✳—✳ Mg^{2+}, 1.0 x 10^{-4} M; ●—● Mg^{2+}, 0.66 x 10^{-4} M.

some variance in the $K_{\mu'}$ values, so that $K_{\mu'}$ increases with increasing concentration of μ'. This variation considerably influenced the magnitude of the dissociation constants, in particular K_3 and K_4, the constants for the interaction of the free metal ion with the enzyme and for the interaction of EM with the substrate, respectively (Table 11). The value of K_6, the constant for the interaction of $MgADP^-$ with the enzyme, was also increased.

Activation of creatine kinase in relation to ADP^{3-} at pH 7.0.

In order to determine the effect of pH on the values for the dissociation constants of enzyme-metal and enzyme-substrate complexes, experiments similar to those mentioned above were carried out at pH 7.0. At this pH it was possible to make a direct comparison with those reported by Kuby et al. (1954b). Plots of $1/v$ against $^1/ADP^{3-}$ at various fixed values of Mg^{2+}, are shown in Fig. 13 and the calculated values for $K_{\mu'}$ and $V_{\mu'}$ in Table 12. No significant variation in the $K_{\mu'}$ values was observed and an average value was taken for the estimation of the dissociation constants. These are included in Table 9. They are again consistent with independent binding of Mg^{2+} and ADP^{3-} to the enzyme.

Activation of creatine kinase by Mg^{2+} in relation to PC.

As the above results had indicated that Mg^{2+} did

TABLE 11

Summary of the dissociation constants for the various reactions leading to the formation of the active enzyme-metal-substrate complex from the data in Table 10. The results shown in column (a) are calculated from the average of the K_μ, values from the first experiment, those in column (b) using the individual values of K_μ, from this experiment and those in column (c) using the average of the individual values of K_μ, from the two experiments.

All values are expressed in terms of M x 10^4.

Dissociation constants	pH 8.0 $K_5 = 2.4$		
	(a)	(b)	(c)
K_1	1.5	2.1	2.3
K_2	1.7	1.7	1.4
K_3	1.8	4.9	7.5
K_4	1.4	0.7	0.4
K_6	1.0	1.5	1.3

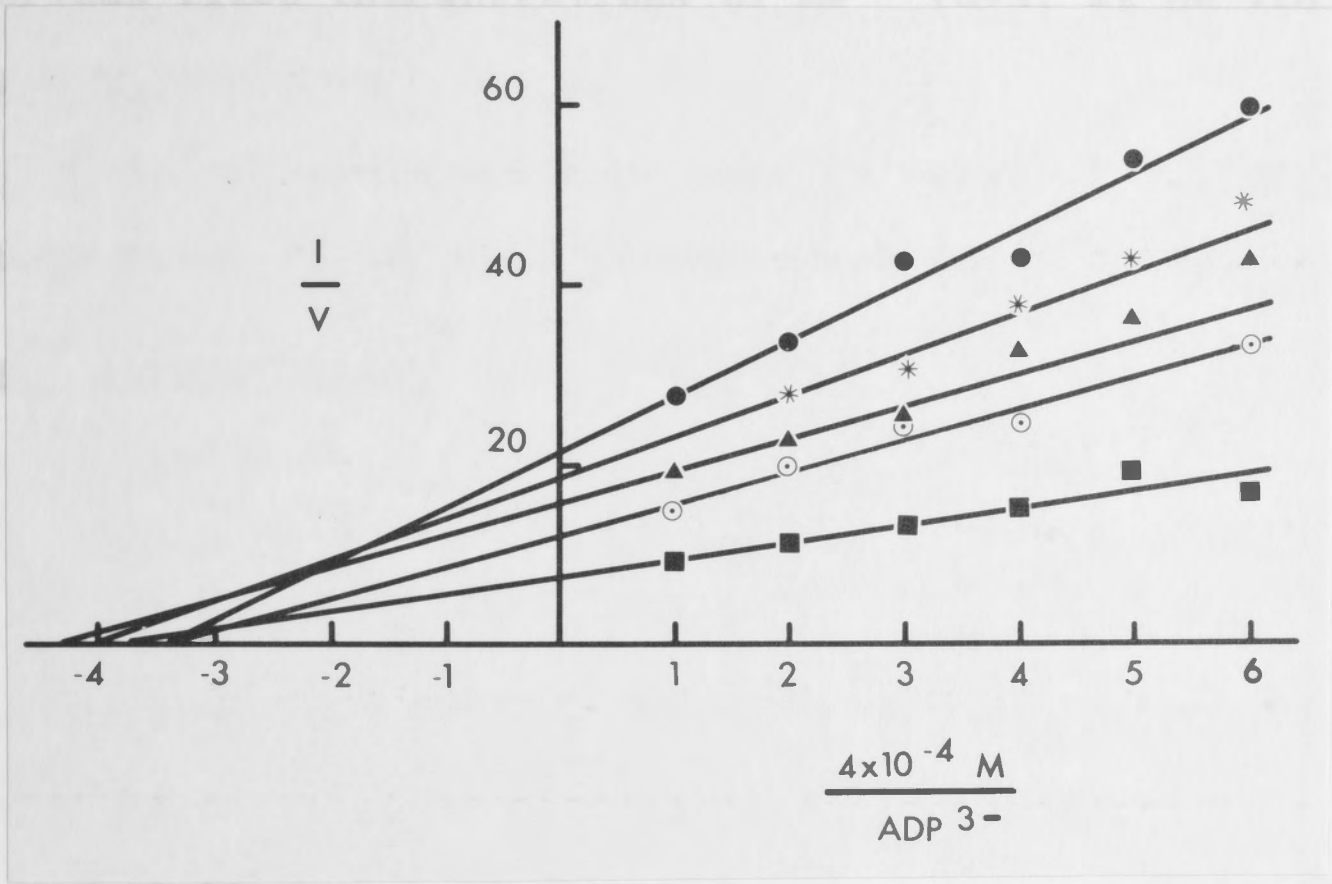


Fig. 13. Effect of the concentration of ADP^{3-} on the initial velocity of the reaction at various fixed concentrations of Mg^{2+}. The concentrations of ADP^{3-} and Mg^{2+} were adjusted by varying the total concentrations of ADP and Mg, with a value of 2,000 M^{-1} for the stability constant of $MgADP^{-}$. The reaction mixtures contained N-ethylmorpholine (pH 7.0), 0.1 M; PC (10^{-2} M) and creatine kinase, 0.9 µg., as well as the indicated amounts of Mg^{2+} and ADP^{3-}. Total volume, 1.0 ml., temp. 30°. Velocity is expressed as µmoles of creatine/µg. of creatine kinase/min. ■—■ Mg^{2+}, 4×10^{-4} M; ⊙—⊙ Mg^{2+}, 2×10^{-4} M; ▲—▲ Mg^{2+}, 1.33×10^{-4} M; ✳—✳ Mg^{2+}, 1×10^{-4} M; ●—● Mg^{2+}, 0.66×10^{-4} M.

TABLE 12

Summary of the values obtained for $K_{\mu'}$ and $V_{\mu'}$ at various fixed concentrations of Mg^{2+} (μ'), at pH 7.0 with K_5 = 0.5 mM.

All K values are expressed in terms of M x 10^4, while those for $V_{\mu'}$ are given in arbitrary units.

Mg^{2+} concentration (μ')	$K_{\mu'}$	$V_{\mu'}$
4.0	1.0	0.84
2.0	1.2	0.52
1.33	0.9	0.40
1.0	1.0	0.33
0.66	1.2	0.29

Average = 1.0

not have an appreciable effect on the binding of ADP^{3-} to the enzyme ($K_1 \approx K_4$), it was conceivable that the function of Mg^{2+} might be to link PC to the enzyme. Thus the metal ion activation was studied in relation to PC at pH 7.0. For this purpose, the concentration of ADP^{3-} was held constant at 1×10^{-3} M while the concentration of PC was varied with Mg^{2+} fixed at various concentrations. The effect of the interaction of Mg^{2+} and PC in reducing the concentration of free Mg^{2+} was regarded, at the time the experiment was carried out, as being small enough to be neglected (see Chapter II) and no consideration was given to the possibility that MgPC could function as a substrate. The latter assumption seemed reasonable in view of the results reported by Kuby et al. (1954b). Fig. 14 shows that the interaction of PC with the enzyme is not influenced by the Mg^{2+} concentration. The K_m value for PC was calculated to be 8×10^{-3} M which may be compared with a value of 1.6×10^{-2} M at pH 8.0. A plot of the reciprocals of the maximum velocities at each value of Mg^{2+} against $1/Mg^{2+}$ yielded a straight line from which the dissociation constant of the enzyme-Mg complex (viz., 2.5×10^{-4} M) was found to be similar to the value of 3.2×10^{-4} M which was obtained at pH 7.0 with free ADP^{3-} as the variable substrate.

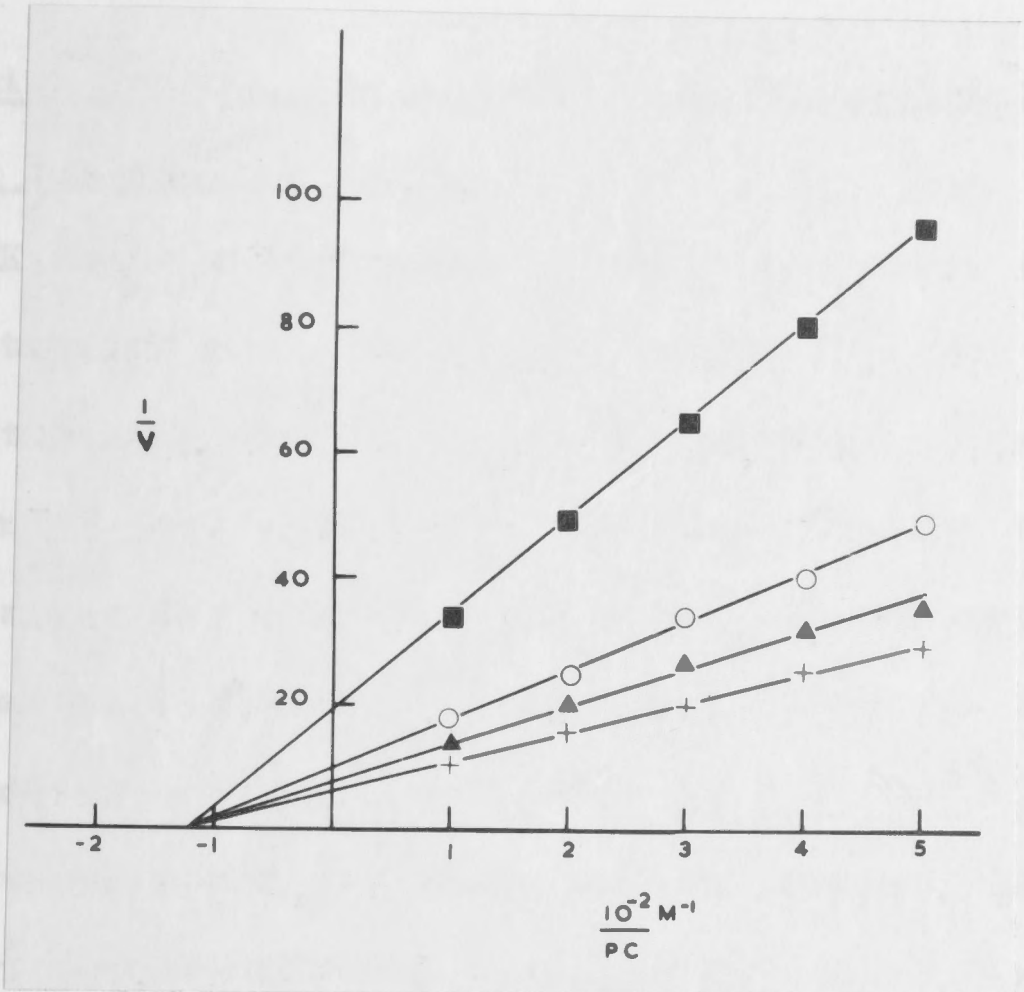


Fig. 14. Effect of the concentration of PC on the initial velocity of the reaction at various fixed concentrations of Mg^{2+}. The concentration of ADP^{3-} was held constant at 10^{-3} M by varying the total concentrations of ADP and Mg. For this purpose a value of 2,000 M^{-1} was used for the stability constant of $MgADP^{-}$. The reaction mixtures contained N-ethylmorpholine (pH 7.0), 0.1 M; ADP^{3-}, 10^{-3} M; creatine kinase, 0.9 μg., and various amounts of PC and Mg^{2+}. Total volume, 1.0 ml., temp. 30°. + — + Mg^{2+}, 4×10^{-4} M; ▲ — ▲ Mg^{2+}, 2×10^{-4} M; ○ — ○ Mg^{2+}, 1×10^{-4} M; ■ — ■ Mg^{2+}, 0.66×10^{-4} M. Velocity expressed as μmoles of creatine/μg. creatine kinase/min.

Determination of the "apparent" K_m values of creatine kinase for Mg^{2+} and ADP.

Kinetic experiments in which the total metal ion concentration had been varied in the presence of a fixed non-saturating amount of ATP had yielded the same apparent K_m value of creatine kinase for the metal ion as obtained for ATP when the total ATP concentration had been varied with the total metal ion fixed at the same non-saturating level (Kuby et al., 1954b). For the reverse reaction, these workers found that there was a two-fold difference in the K_m values for total Mg^{2+} and total ADP, though they did not regard this difference as being significant. Askonas (1952) obtained superimposable lines for plots of 1/v against both $^1/(\text{total } Mg^{2+})$ and $^1/(\text{total ADP})$. Such results are consistent with the idea that the metal-ATP and metal-ADP complexes are the substrates for the enzyme. The results shown in Fig. 15 do not support this contention, though the experiments cannot be regarded as satisfactory because of the scatter of points around the mean lines.

DISCUSSION

At the time that the above experiments were carried out, the determinations of the stability constants of magnesium nucleotide complexes as described in Chapter II had only recently been initiated and some doubt remained

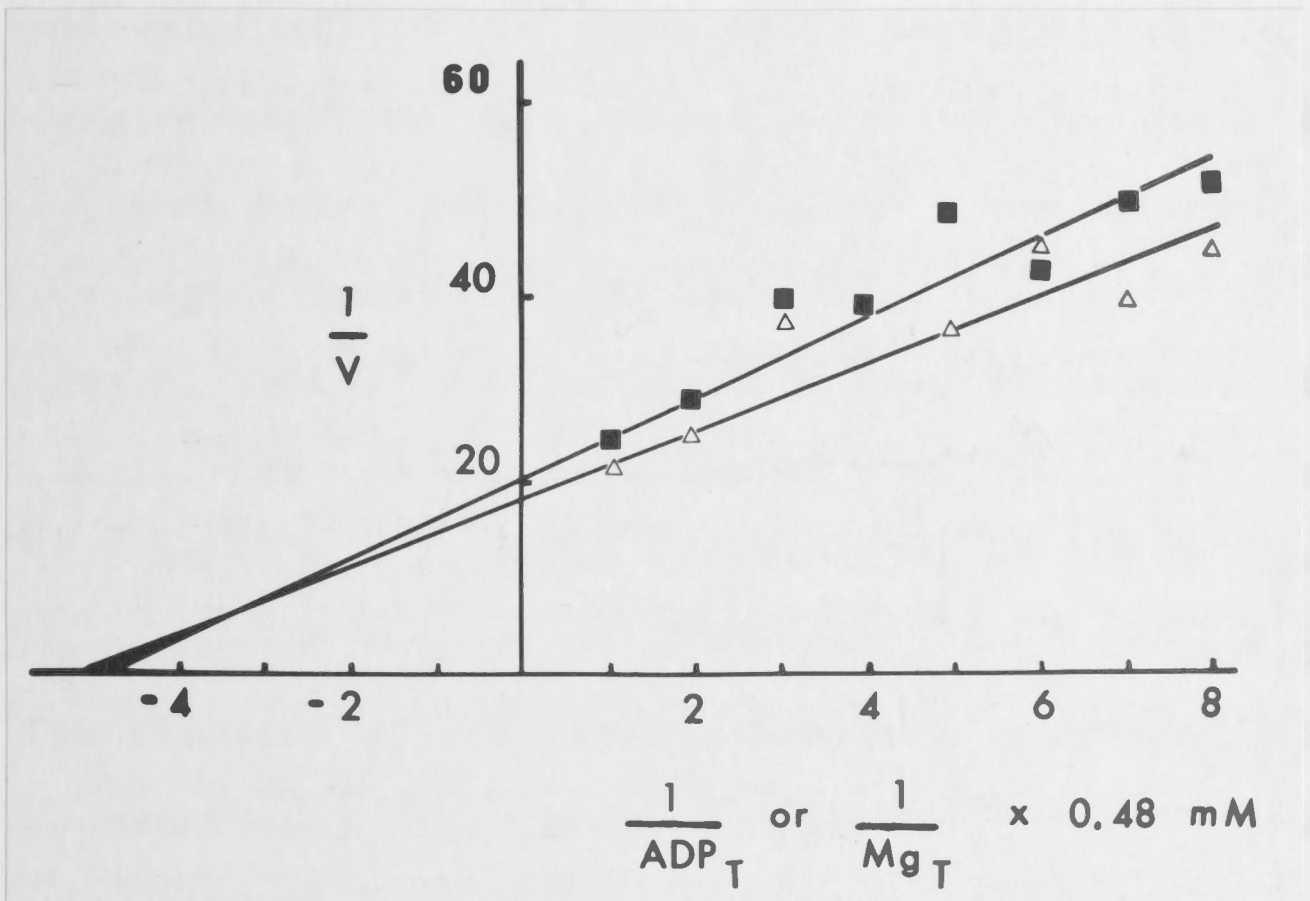


Fig. 15. Apparent K_m values of creatine kinase for Mg and ADP. All reaction mixtures contained N-ethylmorpholine (pH 8.0), 0.1 M; PC, 10^{-2} M and 0.9 µg. of creatine kinase. ■ — ■ , variable component, Mg, total ADP, 2.8×10^{-4} M; △ — △ , variable substrate, ADP, total Mg, 2.8×10^{-4} M. Total volume, 1.0 ml., temp. 30°. Velocity expressed as µmoles of creatine/µg. of creatine kinase/min.

as to the correctness of the value of 4,150 M^{-1} for the stability constant of $MgADP^-$. Thus most of the work in these initial experiments was carried out using the value of 1,000 M^{-1} for the stability constant of this complex and more weight given to the results obtained using this value than for those obtained with the higher constant (cf. Morrison, O'Sullivan and Ogston, 1961). Further studies make it seem more likely that the higher value of the stability constant is correct and that the effect of PC on the apparent magnitude of this constant is small but not negligible. The kinetics of the reverse reaction have since been re-examined and are presented in Chapter VI. However, it should be stressed that the difference in the kinetic results obtained is quantitative rather than qualitative and that the overall rationale, as presented in this chapter, of the kinetic scheme, is not affected by the difference in the stability constants.

In the kinetic calculations two main assumptions were made; that the reactionsleading to the formation of EM, ES and EMS are effectively at equilibrium and that the amounts of EM, ES and EMS formed are too low in relation to those of M, S and MS, to affect appreciably the concentrations of the latter. On this

basis equation (2) was obtained, and it has been shown that this equation adequately expresses the kinetics.

From the agreement of experiment and theory, it might be concluded that all three pathways operate in the formation of the enzyme-Mg-ADP complex. However, equation (2) would be obtained unchanged on the following general assumption: that each of the complex species in the scheme is linked to all others by <u>at least</u> one route, all the reactions of which are rapid enough to maintain equilibrium. This means that it is possible for some of the individual reactions to occur at zero rate without any effect on the steady state kinetics; in particular, any two of the reactions might occur at zero rate provided that <u>both</u> reactions of pathway I, or <u>both</u> reactions of pathway II, or <u>both</u> reactions of pathway III do not occur at zero rate.

From these considerations it is clear that while agreement of the observed kinetics with equation (2) shows that all the species EM, ES and EMS must be taken into consideration, no conclusion can be drawn as to whether any one of the pathways I, II and III is, or is not, operative in forming EMS. In particular, no confirmation that MS is the only substrate can be obtained from the steady state kinetics of this type of system.

In spite of this conclusion, it should be observed that the values of the equilibrium constants obtained must be considered to be valid even if the rates of one or more of the processes to which they apply are negligible or zero. This is true because the same equilibrium concentration of any intermediate must be reached by whatever route equilibrium is obtained and because equilibrium constants are ratios of equilibrium concentrations. Therefore the results with K_5 = 1 mM lead to the conclusion that the energy of binding of ADP^{3-} to the enzyme differs little according to whether Mg^{2+} is, or is not, already combined ($K_1 \approx K_4$); similarly, the binding of Mg^{2+} depends little upon ADP^{3-} ($K_2 \approx K_3$). This makes it unlikely that Mg^{2+} acts as an essential, or even a preferential, link between enzyme and ADP^{3-}. Thus it is unlikely that the role of the metal ion is to act as a bridge by means of which the substrate is linked to the enzyme (cf. Malmström and Rosenberg, 1959; this thesis, Chapter I).

The results with K_5 = 0.24 mM, using the individual K_μ, values (Table 11, columns (b) and (c)), would indicate a slight interaction between Mg^{2+} and ADP^{3-} on the enzyme, i.e., the presence of Mg^{2+} on the enzyme does help bind ADP^{3-} and vice versa. However,

the effect is small and could have been within the experimental error, even though this was reduced to some extent by the use of a statistical method for the calculation of K and V max. values. It should be noted that the value of K_6 for the interaction of $MgADP^-$ with the enzyme (from 1.0 to 1.5 x 10^{-4} M), obtained with K_5 = 0.24 mM, was in better agreement with the value of 1.8 x 10^{-4} M reported by Nihei *et al.* (1961), who used a value of 2,000 M^{-1} for the stability constant of $MgADP^-$ (K_5 = 0.5 mM), than the value of 0.2 x 10^{-4} M obtained using K_5 = 1 mM.

The conclusions drawn from the kinetic data are consistent, at least in part, with the results of the thermodynamic experiments of Kuby and Mahowald (1958). They found that both Mg^{2+} and ATP are independently bound to creatine kinase, although the dissociation constant for the enzyme-Mg complex was over ten times greater than the kinetically determined value reported here. These results for ADP, with K_5 = 1 mM and to a certain extent with K_5 = 0.24 mM, differ from those of Kuby and Mahowald (1958) for ATP in that they found that there was an increased binding of ATP to the enzyme in the presence of Mg^{2+}. Such a result could be due to the non-specific binding of ATP to an inactive enzyme-Mg complex, or possibly to the enzyme

having a greater affinity for $MgATP^{2-}$ than for the free nucleotide.

The strongest kinetic argument for the idea that the creatine kinase reaction occurs by a single compulsory pathway involving the interaction of the enzyme with a Mg-nucleotide complex is the finding that the apparent affinity constant for the substrate at a fixed non-saturating metal concentration is the same as the apparent affinity constant for the metal at the same fixed concentration of substrate (Kuby, Noda and Lardy, 1954b; Askonas, 1952). These results show that under the above conditions, plots of 1/v against 1/(total ADP) and against 1/(total Mg) do not yield superimposable lines. However, it is of interest to note that Dixon and Webb (1958b) have pointed out that this result would also be obtained if an enzyme-metal-substrate complex were formed by all three pathways indicated above and if the K_m values for the enzyme-metal and enzyme-substrate complexes were identical.

Studies of the activation of creatine kinase by Mg^{2+} in relation to PC indicated that the divalent metal ion is not involved in the linkage of this substrate to the enzyme. Thus as far as pathways I and II are concerned, it would seem that Mg^{2+} could be involved directly or indirectly in the formation

of a transition state complex involving both PC and ADP. The possible nature and function of such a complex is discussed in the Conclusion (Chapter VIII).

SUMMARY

A kinetic approach has been used to evaluate the dissociation constants for all the possible intermediate steps in the formation of an enzyme-Mg-substrate complex for the reverse reaction catalysed by creatine kinase. The results suggest that Mg^{2+} and the two substrates are capable of being bound independently to the enzyme and that Mg^{2+} does not greatly affect the binding of the substrates.

CHAPTER V

THE INHIBITION BY EXCESS Mg^{2+} AND EXCESS ADP OF THE REVERSE REACTION CATALYSED BY CREATINE KINASE

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INTRODUCTION

On the basis that rapid equilibrium conditions prevailed and that the species could be bound independently to the enzyme, the kinetic results presented in Chapter IV had indicated that the reaction of the various species, Mg^{2+}, ADP^{3-} and $MgADP^{-}$ with creatine kinase could be operative in the formation of an active EMS complex. If such a conclusion were correct, it might be expected that both Mg^{2+} and ADP^{3-} would compete with $MgADP^{-}$ for the same site on the enzyme. In other words, in the presence of $MgADP^{-}$, an excess of either Mg^{2+} or ADP^{3-} should give rise to competitive inhibition.

In a previous study of the inhibition of the reverse reaction, under conditions where the concentration of ADP was held constant while the Mg^{2+} concentration was increased, Kuby, Noda and Lardy (1954b) found that the reaction velocity passed through a maximum and then decreased. With this approach it would not be possible to determine the nature of the inhibition and furthermore,

if there were competition between Mg^{2+} and $MgADP^-$ for the same site, such inhibition would be small, as the enzyme would be saturated with $MgADP^-$ before Mg^{2+} was in excess.

This chapter describes a study of the inhibition of creatine kinase by Mg^{2+} and ADP^{3-}, under conditions where the concentration of the inhibitor was maintained at a number of fixed levels, while the $MgADP^-$ concentration was varied within a non-saturating range of concentrations. In addition, studies of the effect of various non-saturating concentrations of PC on the inhibition have been carried out.

THEORY

Inhibition of creatine kinase by Mg^{2+} with respect to $MgADP^-$.

The initial velocity equation (Chapter IV) may be rearranged in terms of μ (Mg^{2+}) and z ($MgADP^-$), so that

$$v = \frac{ke}{\frac{K_6}{z} + \frac{K_6}{K_3} \cdot \frac{\mu}{z} + \frac{K_2}{\mu} + 1} \qquad (1)$$

The findings of Kuby *et al.* (1954b) and Nihei *et al.* (1961) had indicated that PC reacted with the enzyme independently of the nucleotide. Thus, assuming that the enzyme-PC complex has a dissociation constant, K_7, which is independent of the form of the enzyme, equation (1) may

be modified to take into account the effect of the PC concentration on the reaction velocity, viz.,

$$v = \frac{ke}{\left\{1 + \frac{K_7}{[PC]}\right\}\left\{\frac{K_6}{z} + \frac{K_6}{K_3} \cdot \frac{\mu}{z} + \frac{K_2}{\mu} + 1\right\}} \qquad (2)$$

In the inverse form

$$\frac{1}{v'} = \frac{K_6}{ke} \cdot \frac{1}{z} \cdot \left\{1 + \frac{\mu}{K_3}\right\} + \frac{1}{ke}\left\{\frac{K_2}{\mu} + 1\right\} \qquad (3)$$

where

$$\frac{1}{v} = \frac{1}{v'} \cdot \left\{1 + \frac{K_7}{[PC]}\right\} \qquad (4)$$

Plots of $1/v'$ against $1/z$, with μ held constant at various fixed values, μ' and PC held constant, should give a family of straight lines with slope and maximum velocity varying with the concentration of μ and the intercepts on the x- and y-axis defined by

$$V_{\mu'} = \frac{ke}{\frac{K_2}{\mu'} + 1} \quad \text{and} \quad K_{\mu'} = \frac{K_6\left(1 + \frac{\mu'}{K_3}\right)}{\frac{K_2}{\mu'} + 1}$$

For the condition, $\mu \gg K_2$, equation (3) reduces to

$$\frac{1}{v'} = \frac{K_6}{ke} \cdot \frac{1}{z}\left\{1 + \frac{\mu}{K_3}\right\} + \frac{1}{ke} \qquad (5)$$

so that plots of $1/v'$ against $1/z$, for various fixed values of μ' and PC, should give a series of straight lines with the same value of $V_{\mu'}$ and with $K_{\mu'}$ dependent on μ', according to the relationship

$$K_{\mu'} = K_6 \left(1 + \frac{\mu'}{K_3}\right) \qquad (6)$$

Inhibition of creatine kinase by ADP^{3-} with respect to $MgADP^{-}$.

Rearranging the general equation in terms of σ (ADP^{3-}) and z, and allowing for the interaction of PC with the enzyme,

$$v = \frac{ke}{\left\{1 + \frac{K_7}{[PC]}\right\}\left\{\frac{K_6}{z} + \frac{K_6}{K_1} \cdot \frac{\sigma}{z} + \frac{K_4}{\sigma} + 1\right\}} \qquad (7)$$

$$\text{and} \quad \frac{1}{v'} = \frac{K_6}{ke} \cdot \frac{1}{z}\left\{1 + \frac{\sigma}{K_1}\right\} + \frac{1}{ke}\left\{\frac{K_4}{\sigma} + 1\right\} \qquad (8)$$

$$\text{with} \quad \frac{1}{v} = \frac{1}{v'}\left\{1 + \frac{K_7}{[PC]}\right\} \qquad (4a)$$

Thus plots of $1/v'$ against $1/z$, with σ held constant at σ', and PC constant, should be linear, with

$$V_{\sigma'} = \frac{ke}{\frac{K_4}{\sigma'} + 1} \quad \text{and} \quad K_{\sigma'} = \frac{K_6\left(1 + \frac{\sigma'}{K_1}\right)}{\frac{K_4}{\sigma'} + 1}$$

with both $V_{\sigma'}$ and $K_{\sigma'}$ dependent on σ'.

For the condition, $\sigma \gg K_4$, equation (8) becomes

$$\frac{1}{v'} = \frac{K_6}{ke} \cdot \frac{1}{z}\left\{1 + \frac{\sigma}{K_1}\right\} + \frac{1}{ke} \qquad (9)$$

so that plots of $1/v'$ against $1/z$, for fixed values of σ' and PC, should give a series of straight lines with the same V and with K dependent on σ', according to the relationship

$$K_{\sigma'} = K_6\left(1 + \frac{\sigma'}{K_1}\right) \qquad (10)$$

MATERIALS AND METHODS

When experiments were carried out with concentrations of Mg^{2+} greater than those used previously, a time-dependent denaturation of the enzyme was observed (cf. Chapter III). It was found that EDTA not only prevented this denaturation but also considerably enhanced the activity of the enzyme. Therefore, EDTA at a concentration of 10^{-5} M, was added to all reaction mixtures. This concentration of EDTA was considered to have only a negligible effect on the concentration of free Mg^{2+}.

All materials and methods were as described previously, except that for the experiments with excess Mg^{2+}, the concentration of EDTA in the alkali solution used to stop the reaction was increased to prevent the precipitation of $Mg(OH)_2$, the concentration of EDTA in the final mixture being 0.03 M.

The apparent stability constant of $MgADP^-$ is a function of the PC concentration (see Chapter II) and this was taken into account when calculating the total Mg^{2+} and total ADP concentrations required at the different non-saturating levels of PC used. All experiments were run for two time periods (usually $\frac{3}{4}$ and

$1\frac{1}{2}$ minutes), to ensure that initial velocities were being observed.

The terminology suggested by Cleland (1963) has been used to classify the various types of inhibition obtained and his procedures have also been used to estimate the various inhibition constants (K_i). Thus the inhibition is called competitive, uncompetitive and non-competitive, respectively, when the slope, vertical intercept, or both, are a function of the inhibitor concentration. If the slope and/or intercept is a linear function of the inhibitor concentration, the inhibition is called linear. The K_i values for linear inhibitions have been obtained by replotting slopes and/or intercepts against inhibitor concentration.

The weighted mean squares method of Wilkinson (1961) was used to calculate the maximum velocity and K values from the double reciprocal plots.

RESULTS

Preliminary experiments, carried out at the same time as the kinetic results reported in Chapter IV, had shown that inhibition occurred with excess Mg^{2+} or excess ADP. When the concentration of $MgADP^-$ present was calculated using the value of 1,000 M^{-1} for the stability constant of this complex, it became apparent that the reaction velocity decreased as the concentration

of $MgADP^-$ increased and that the velocity was greater in the presence of excess Mg^{2+} than when ADP^{3-} was in excess.

These results were reported by Morrison, O'Sullivan and Ogston (1961) and Table 13 is reproduced from this publication. It appeared that the results could be reasonably accounted for by the general reaction scheme. Thus, using the values obtained with K_5 = 1 mM (Table 9, Chapter IV), the calculated ratios of the velocities 1a/1b, 2a/2b, 3a/3b were 0.84, 0.74 and 0.65, respectively, while the observed values were 0.86, 0.67 and 0.56. However, this simple calculation did not give good correlation with the observed results, when the constants obtained with the higher stability constant (K_5 = 0.24 mM) were used. The calculated ratios using the constants from column (b), Table 11, Chapter IV (i.e., those obtained using the individual values of K_{μ},, these constants being similar to those reported in the reassessment of the reverse reaction in Chapter VI) were 0.86, 0.83 and 0.82.

<u>The inhibition of creatine kinase by Mg^{2+} with respect to $MgADP^-$.</u>

The concentration of free Mg^{2+} was maintained at 1, 5, 10 and 15 mM and the concentration of $MgADP^-$ was varied from 0.8 mM to 0.065 mM. Under these conditions, except when the concentration of Mg^{2+} was 1 mM, where

TABLE 13

Correlation of the reaction velocities with the concentrations of Mg^{2+}, ADP^{3-} and $MgADP^{-}$

The reaction mixtures contained N-ethylmorpholine (pH 8.0), 0.1 M; PC, 10^{-2} M and creatine kinase, 0.9 µg., as well as the indicated amounts of Mg and ADP. Vol., 1.0 ml., temp. 30°. The concentrations of Mg^{2+}, ADP^{3-} and $MgADP^{-}$ were calculated on the basis that K_5 = 1 mM. All concentrations are expressed as mM and the reaction velocities are given in terms of µM of creatine/µg. creatine kinase/min.

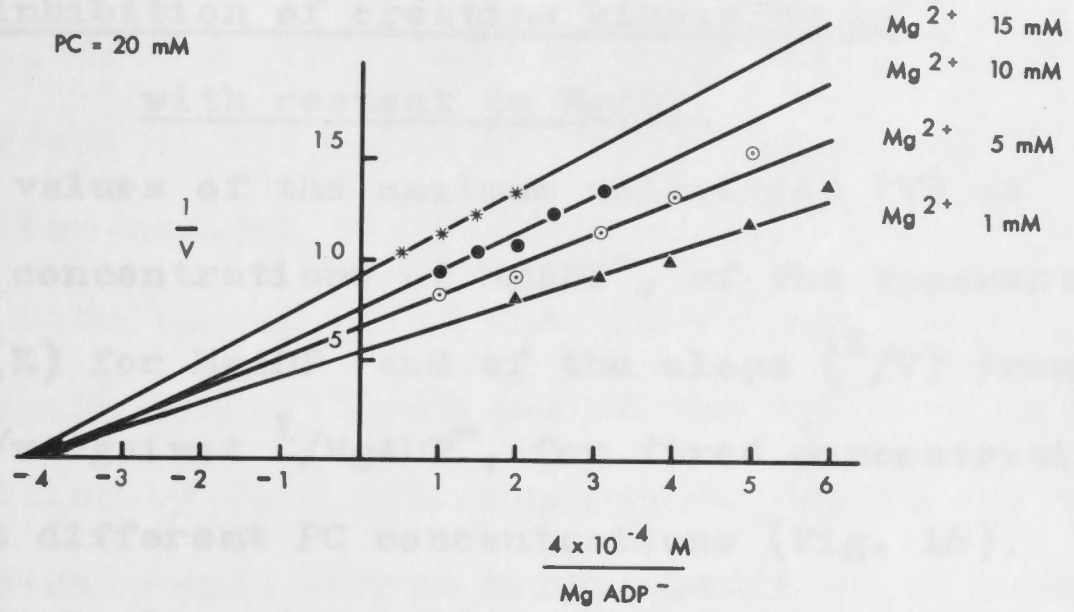
Total Mg	Total ADP	$MgADP^{-}$	Mg^{2+}	ADP^{3-}	Velocity (µM creatine/µg. creatine kinase/ min.)
1. (a) 1	2.5	0.65	0.35	2.15	0.50
(b) 2.5	1	0.65	2.15	0.35	0.59
2. (a) 1	5	0.82	0.18	4.16	0.41
(b) 5	1	0.82	4.16	0.18	0.61
3. (a) 1	10	0.9	0.1	9.1	0.24
(b)10	1	0.9	9.1	0.1	0.43

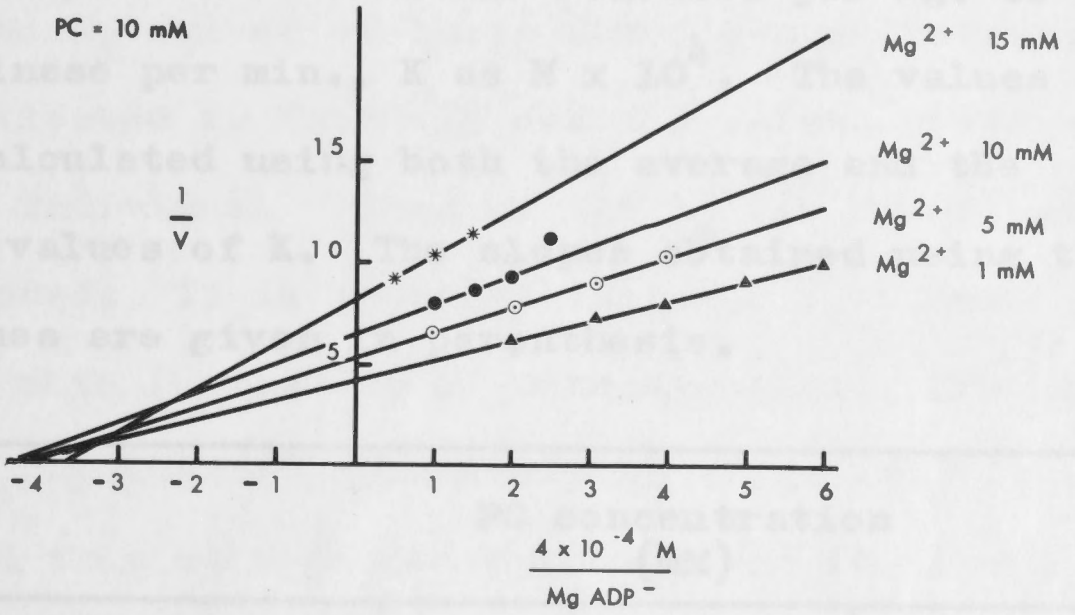
the ADP^{3-} concentration ranged from approximately 0.5 to 0.1 of K_1, the concentration of ADP^{3-} was negligible compared to its K_m value (K_1).

Plots of $^1/v$ against $^1/MgADP^-$, at the above four levels of Mg^{2+} and with PC at concentrations of 20, 10 and 5 mM, are shown in Fig. 16. It is clear that the inhibition increases with increasing Mg^{2+} concentration, though the Mg^{2+} concentration required to obtain inhibition was much greater than would have been expected on the basis of K_3 being approximately $1\text{-}3 \times 10^{-4}$ M. Further, the inhibition appears to be of the non-competitive type.

The calculated values of the K_m for $MgADP^-$ and the maximum velocities at saturating concentrations of $MgADP^-$, from these plots, have been collected in Table 14, together with the values for the slopes of the lines. The K_m values for $MgADP^-$, at each concentration of PC, appear to vary in a random fashion. The average value of the K_m, at each concentration of PC, varied slightly with the PC concentration, but it is doubtful if this is of any significance. The average values of the K_m were used to calculate the slope, but for comparative purposes, values were also obtained by using the individual estimates of K_m and these are included in Table 14.

Fig. 16. The inhibition of creatine kinase by Mg^{2+} with respect to $MgADP^-$, at different concentrations of PC. The concentrations of Mg^{2+} and $MgADP^-$ were adjusted by varying the total concentrations of ADP and Mg, using values of 3,300 M^{-1}, 3,600 M^{-1} and 3,800 M^{-1} for the stability constant of $MgADP^-$ at PC equal to 20, 10 and 5 mM respectively. The reaction mixtures contained N-ethylmorpholine (pH 8.0), 0.1 M; creatine kinase, 0.576 µg. and EDTA (10^{-5} M) as well as the indicated amounts of Mg^{2+}, $MgADP^-$ and PC. Total volume, 1.0 ml., temp. 30°. Velocity is expressed as µmoles of creatine/µg. of creatine kinase/min.


PC = 20 mM
1/V
15
10
5
-4
-3
-2
-1
1
2
3
4
5
6
4 x 10 -4 M
Mg ADP -
Mg 2+ 15 mM
Mg 2+ 10 mM
Mg 2+ 5 mM
Mg 2+ 1 mM


PC = 10 mM
1/V
15
10
5
-4
-3
-2
-1
1
2
3
4
5
6
4 x 10 -4 M
Mg ADP -
Mg 2+ 15 mM
Mg 2+ 10 mM
Mg 2+ 5 mM
Mg 2+ 1 mM

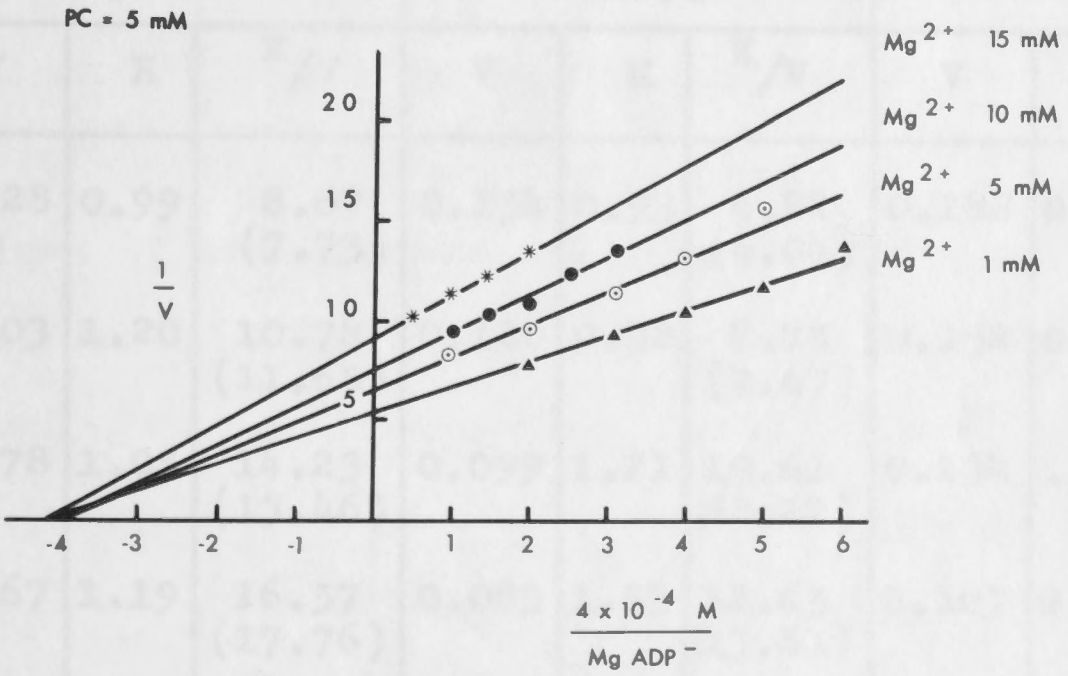

PC = 5 mM
1/V
20
15
10
5
-4
-3
-2
-1
1
2
3
4
5
6
4 x 10 -4 M
Mg ADP -
Mg 2+ 15 mM
Mg 2+ 10 mM
Mg 2+ 5 mM
Mg 2+ 1 mM

TABLE 14

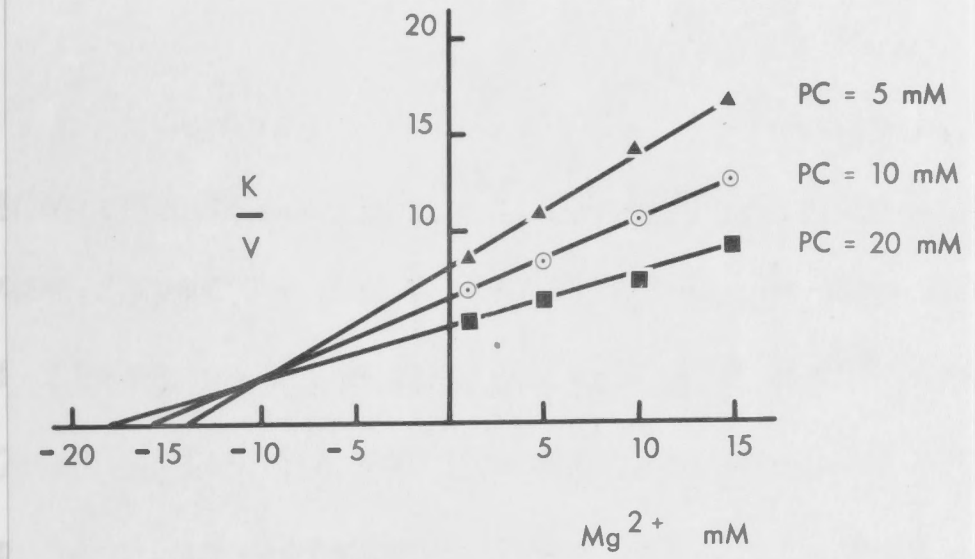
The inhibition of creatine kinase by Mg^{2+} with respect to $MgADP^-$

Calculated values of the maximum velocities (V) at saturating concentrations of $MgADP^-$, of the apparent K_m values (K) for $MgADP^-$ and of the slope (K/V) from the plots of $1/v$ against $1/MgADP^-$, for fixed concentrations of Mg^{2+}, at different PC concentrations (Fig. 16). V is expressed in μmoles of creatine released per μg. of creatine kinase per min., K as M x 10^4. The values of K/V were calculated using both the average and the individual values of K. The slopes obtained using the latter values are given in parenthesis.

Mg^{2+} conc. (mM)	PC concentration (mM)								
	5.0			10.0			20.0		
	V	K	K/V	V	K	K/V	V	K	K/V
1.0	0.128	0.99	8.67 (7.73)	0.154	0.93	6.82 (6.04)	0.188	0.95	5.16 (5.05)
5.0	0.103	1.20	10.78 (11.65)	0.120	0.92	8.75 (7.67)	0.152	0.99	6.38 (6.51)
10.0	0.078	1.05	14.23 (13.46)	0.099	1.21	10.61 (12.22)	0.134	1.03	7.24 (7.69)
15.0	0.067	1.19	16.57 (17.76)	0.083	1.13	12.65 (13.61)	0.107	0.88	9.07 (8.22)
Average K_m for $MgADP^-$		1.11			1.05			0.97	

Estimates of the inhibition constant, K_i, for Mg^{2+} were obtained from plots of both the slope and the reciprocal of the maximum velocities against Mg^{2+} concentration at each concentration of PC (Fig. 17a, 17b). As both the slope of the lines and the intercepts are linear functions of the Mg^{2+} concentration, the inhibition by Mg^{2+} with respect to $MgADP^-$ may be classified as simple linear non-competitive (Cleland, 1963). The K_i values at three fixed concentrations of PC are collected in Table 15 and the values obtained using the individual values of the K_m for $MgADP^-$ are also included. It is apparent that the K_i value increases with increasing PC concentration. From a plot of K_i against PC concentration (Fig. 18a) it was determined that at zero concentration of PC, the K_i value for Mg^{2+} was 12.8 mM. Alternatively, a plot of $^1/K_i$ against $^1/PC$ (Fig. 18b) gave a value of 20.3 mM at infinite PC concentration.

Values of the maximum velocity (Vo) at saturating concentrations of $MgADP^-$ and extrapolated to zero concentration of Mg^{2+}, were also obtained from Fig. 17b for each concentration of PC and are included in Table 15. A plot of $^1/Vo$, at each concentration of PC, against $^1/PC$, according to equation (4) gave a K_m value for PC of 3.6 mM (Fig. 19). This may be compared with the values



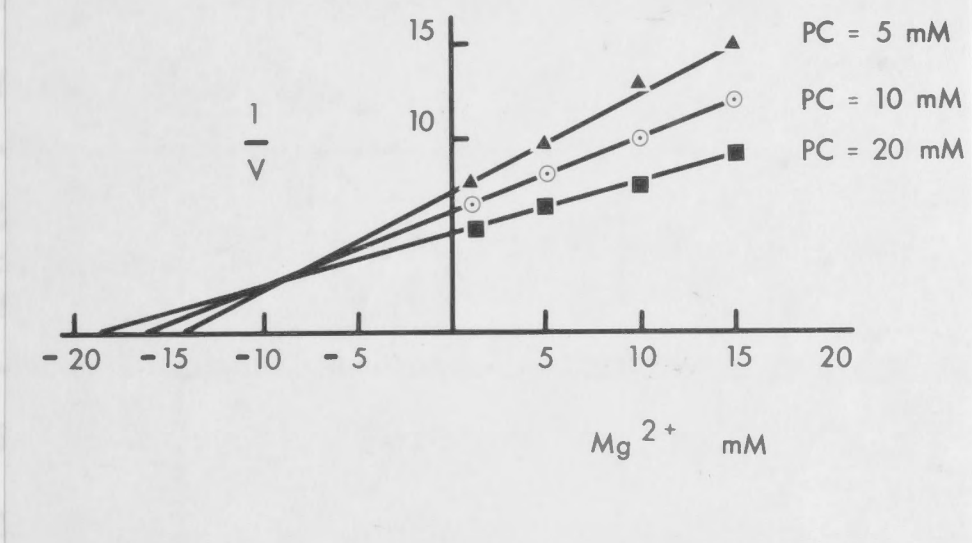


Fig. 17.(a). Plot of slope (K/V) against Mg^{2+} concentration at different PC concentrations. Data obtained from the plots for the inhibition by Mg^{2+} with respect to $MgADP^-$ (Fig. 16).

(b). Plot of intercept (1/V) against Mg^{2+} concentration at different PC concentrations. Data obtained from the plots for the inhibition by Mg^{2+} with respect to $MgADP^-$ (Fig. 16).

Lines of best fit were drawn using the method of least squares.

TABLE 15

Variation of K_i (Mg^{2+}) with PC concentration

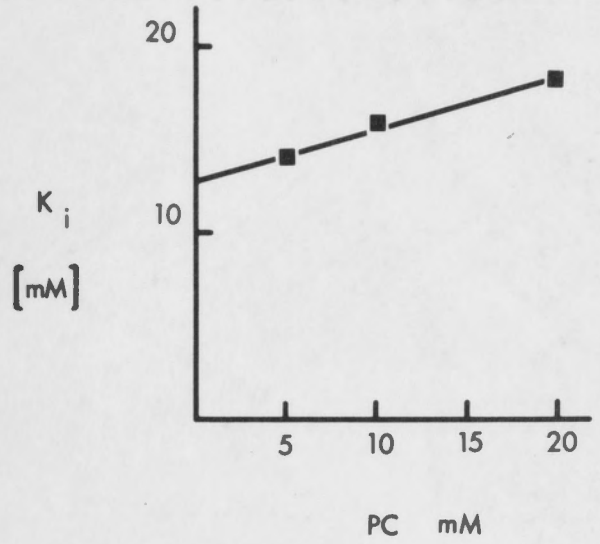
The values reported were obtained from the secondary plots of slope and intercept against Mg^{2+} concentration (Fig. 17a, 17b). Vo is the maximum velocity for each PC concentration as obtained from Fig. 17a and is expressed as μmoles creatine/μg. creatine kinase/min. Values of K_i in parenthesis were obtained using the individual K values from Table 14.

PC concentration (mM)	K_i (intercept) (mM)	K_i (slope) (mM)	Vo
5	14.0	14.0 (11.0)	0.137
10	16.0	15.9 (9.3)	0.161
20	18.4	18.2 (22.8)	0.188

Extrapolation of K_i values to zero concentration of PC (Fig. 18a): K_i = 12.8 mM.

Plot of $^1/K_i$ against $^1/PC$ (Fig. 18b):

K_i = 20.3 mM at PC = ∞



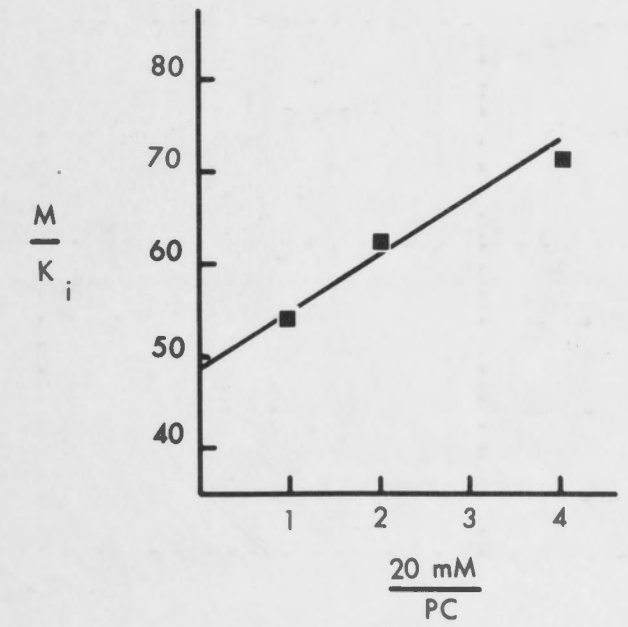


Fig. 18.(a) Plot of K_i values, for the inhibition by Mg^{2+} with respect to $MgADP^-$, at different PC concentrations, against PC concentration.

(b) Plot of $1/K_i$, for the inhibition by Mg^{2+} with respect to $MgADP^-$, at different PC concentrations, against 1/PC.

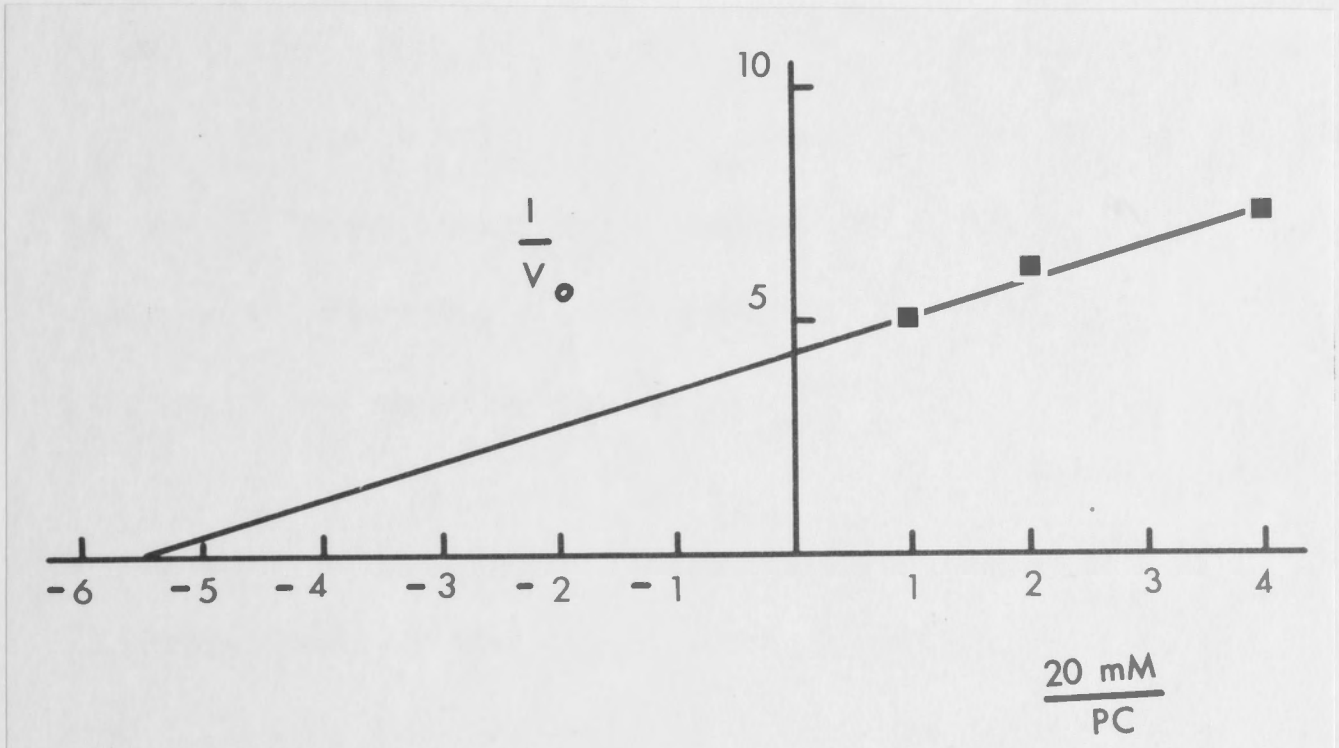


Fig. 19. Plot of $^{1}/Vo$ values, at different PC concentrations, from the inhibition by Mg^{2+} with respect to $MgADP^{-}$, against the reciprocal of the PC concentration.

of 16 mM, 0.8 mM and 5 mM reported by Morrison et al. (1961), Nihei et al. (1961) and Kuby et al. (1954b), respectively. The plot shown in Fig. 19 also gave the value of 0.23 μmoles creatine/μg. creatine kinase/min. (18,600 moles/min./mole of creatine kinase) for the maximum velocity at pH 8.0 when the enzyme is saturated with $MgADP^-$ and PC, and the Mg^{2+} concentration is zero.

On the basis of the theory previously elaborated, it would have been expected that both $V_{\mu'}$ and $K_{\mu'}$ would vary with varying μ' (equation 3). As $K_{\mu'}$ is constant, it could be concluded that $\left\{\frac{K_2}{\mu'} + 1\right\} = \left\{1 + \frac{\mu'}{K_3}\right\}$, that is $K_2K_3 = \mu'^2$, which is untenable as μ' is a variable. Alternatively, the requirement could be met if $\mu' \gg K_2$ and $K_3 \gg \mu'$, but such relationships would have given K_m and maximum velocities which were independent of μ'. As the maximum velocity is a function of the concentration of μ', it appears that the postulated kinetic scheme cannot satisfactorily explain the inhibition by Mg^{2+}.

A consideration of some factors which might contribute to the inhibition observed with excess $MgCl_2$.

Non-competitive inhibition, insofar as the inhibitor does not react at the active site, may arise from some indirect effect on the reaction velocity. In particular, the conversion of substrate to an inactive

form, as a result of reaction with an inhibiting agent, can give rise to an apparent non-competitive inhibition. A number of possible factors which might lead to non-competitive inhibition were examined.

Formation of Mg_2ADP^+: It had been previously suggested (Kuby et al., 1954b) that the inhibition of creatine kinase by excess metal ion could be due to the formation of a second complex; viz., Mg_2ADP^+, which was not active in the enzymic reaction. There has been little evidence for the formation of such a complex, although Burton (1959) has concluded that it could form. He suggested an upper limit of 70 M^{-1} for K^*, where

$$K^* = \frac{[Mg_2ADP^+]}{[Mg^{2+}][MgADP^-]}$$

Consideration was given to the possible formation of this complex under conditions where the concentration of Mg^{2+} was much greater than that of $MgADP^-$. The effect of assigning values to K^*, within a narrow range, on the reciprocal plots, was tested. Table 16 shows the calculated values of Mg_2ADP^+ at an apparent $MgADP^-$ concentration of 0.2 mM, together with the corrected concentrations of $MgADP^-$, for K^* at 20 M^{-1} and 50 M^{-1}. In Fig. 20, the data obtained for the inhibition of the enzyme by Mg^{2+} at PC equal to 5 mM have been replotted

TABLE 16

The possible effect of the formation of Mg_2ADP^+ on the concentration of $MgADP^-$

Calculated values of Mg_2ADP^+ and the resultant value of $MgADP^-$ for an apparent concentration of $MgADP^-$ = 0.2 mM, with assumed values of 20 M^{-1} and 50 M^{-1}, for K^*, where $K^* = \frac{[Mg_2ADP^+]}{[Mg^{2+}][MgADP^-]}$. Results with K^* = 50 M^{-1} are shown in parenthesis.

Mg^{2+} (mM)	1	5	10	15
Mg_2ADP^+ (mM)	0.004 (0.010)	0.018 (0.040)	0.033 (0.067)	0.046 (0.086)
$MgADP^-$ (mM)	0.196 (0.190)	0.182 (0.160)	0.167 (0.133)	0.154 (0.114)

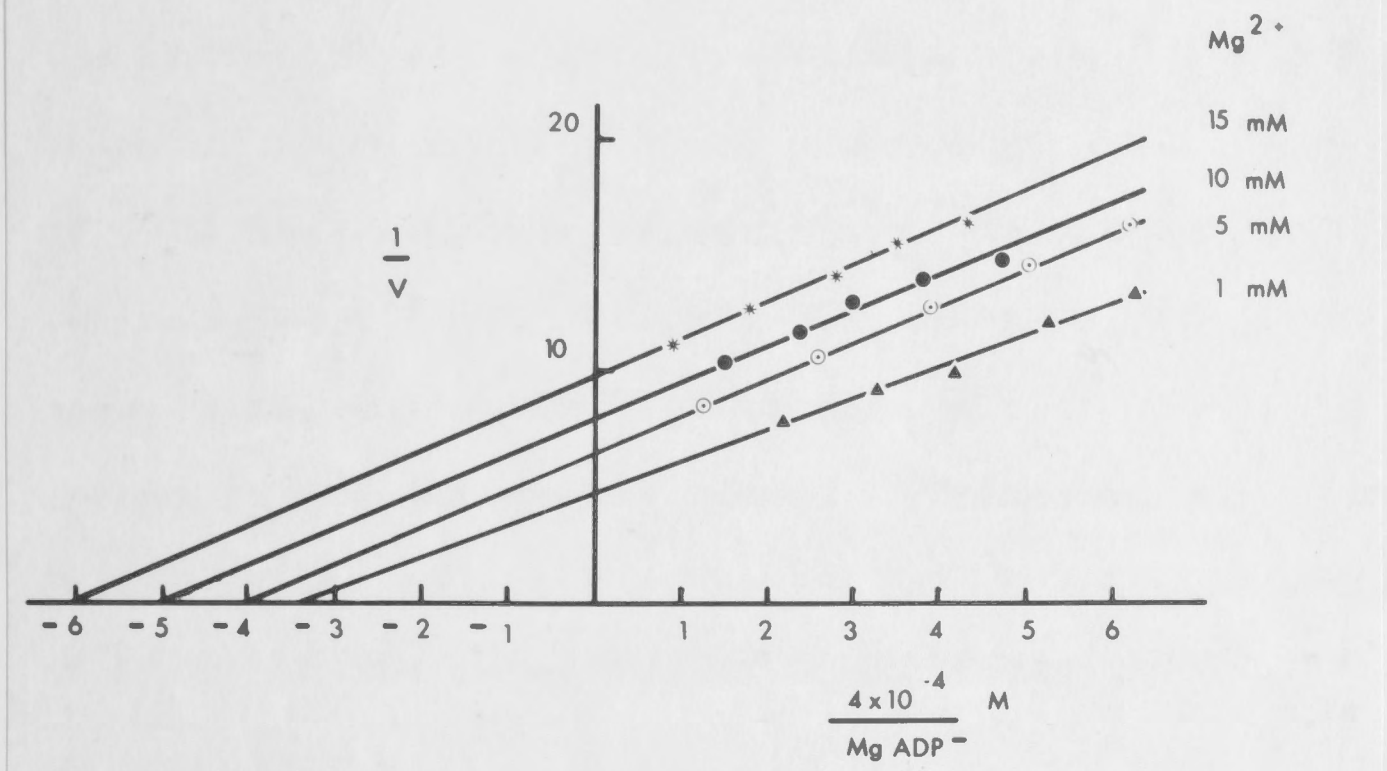


Fig. 20. Data for inhibition of $MgADP^-$ by Mg^{2+} as in Fig. 16 at PC = 5 mM replotted taking the possible existence of $Mg_2\ ADP^+$ into account, with $K^* = 50\ M^{-1}$ where $K^* = \frac{[Mg_2ADP^+]}{[Mg^{2+}][MgADP^-]}$.

on the basis of $K^* = 50\ M^{-1}$. The result is that the inhibition becomes linear uncompetitive. A plot of the reciprocal of the maximum velocity, at different Mg^{2+} concentrations, against Mg^{2+} concentration, showed that the intercept was a linear function of Mg^{2+} and a K_i value of approximately 12 mM was calculated. This set of data has also been tested for K^* values of 20, 100, 200 and 500 M^{-1} with effectively the same result in each case, i.e., uncompetitive inhibition with little or no change in the intercept values. (Theoretically, the values of the intercepts should have been independent of K^*.) If the formation of Mg_2ADP^+ was the sole contributing factor to inhibition, it would have been expected that all the points would fall on the same straight line. As this does not occur, the formation of Mg_2ADP^+ does not satisfactorily account for the observed inhibition.

Formation of MgPC: The results of Kuby et al. (1954b) had indicated that free PC, rather than an MgPC complex, was the substrate for the enzymic reaction. Thus, at relatively high concentrations of Mg^{2+}, the formation of MgPC could lead to an apparent inhibition. The data at PC = 10 mM was tested using the value of 12 M^{-1} reported in Chapter II for the stability constant of MgPC.

The formation of this complex would reduce the concentrations of both PC and Mg^{2+}, and hence of $MgADP^-$.

The effect on the $MgADP^-$ concentration was negligible, so that the plots of $^1/v$ against $^1/MgADP^-$ (Fig. 16) were unaffected. However, the values of K_i for Mg^{2+}, obtained from the secondary plots, were altered by about 10%. Thus the K_i obtained from the plot of slope against Mg^{2+} (Fig. 17b) was changed from 15.9 mM to 14.0 mM. As the concentrations of both Mg^{2+} and PC were altered, the extrapolated value of K_i obtained by plotting apparent K_i against PC concentration (Fig. 18a, Table 15) was virtually unaffected. The K_m value for PC was also slightly reduced (see next section).

Ionic strength: Kistiakowsky and Shaw (1953) showed that the activity of urea amidohydrolase (urease; 3.5.1.5) decreased as the ionic strength increased, independent to the nature of the electrolyte which was added. They showed that it was possible to fit the Debye-Hückel equation for the ionic activity coefficients

$$\frac{\sqrt{\mu}}{\log(V^\circ/V)} = \frac{1}{A} + \frac{B}{A} \cdot \sqrt{\mu}$$

(μ = ionic strength; V = velocity; V° = velocity at zero ionic strength; and A and B the constants for the Debye-Hückel limiting law). Thus, a plot of $\sqrt{\mu}/\log(V^\circ/V)$ against $\sqrt{\mu}$ was linear and values could be assigned to

A and B.

In the inhibition experiments reported in this section, the ionic strength increased from approximately 0.11 at Mg^{2+} = 1 mM to approximately 0.15 at Mg^{2+} = 15 mM. Using the V values in Table 14 an estimate of V° was obtained and used in an attempt to fit the Debye-Hückel relationship. However, the plots were hyperbolic and no estimates of A and B could be obtained.

Efforts were also made to test the effect of ionic strength experimentally by adding NaCl and KCl. These electrolytes inhibited the reaction to a lesser degree than $MgCl_2$ at the same ionic strength and the observed inhibition could have been due to the formation of complexes such as $NaADP^{2-}$ and $KADP^{2-}$. Tetraethylammonium ion, which does not interact with the nucleotide (Smith and Alberty, 1956) was added as the bromide. This salt was found to be a strong inhibitor of creatine kinase and appeared to be partially competitive with respect to PC.

Another effect of increasing the ionic strength would be to disturb the equilibrium between $MgADP^{-}$ and Mg^{2+} and ADP^{3-}. Thus, Burton (1959) demonstrated that the apparent stability constant, and thus the concentration of $MgADP^{-}$, decreased with increasing ionic strength.

Effect of chloride ion: It was reported by Nihei et al. (1961) that a number of anions, including Cl^-, were competitive inhibitors of creatine kinase with respect to PC. They reported a K_i for Cl^- of 0.11 M at pH 6.3, though, as the chloride was added as NaCl, it was not clear that the observed inhibition was entirely due to Cl^-, rather than to Na^+, and thus the figure of 0.11 M probably represents an upper limit.

As Mg^{2+} was added as $MgCl_2$, the effect of Cl^- as a non-competitive inhibitor with respect to $MgADP^-$ was tested on the data presented in Table 14 at PC = 5 mM. The Vo value at this concentration of PC was 0.137 µmoles creatine/µg. creatine kinase/min. (Table 15). Using the formula

$$V = \frac{V^*}{1 + \frac{i}{K_i}}$$

the V^* value at zero concentration of Cl^- was calculated to be 0.171 µmoles creatine/µg. creatine kinase/min., the concentration of Cl^- in 0.1 M N-ethylmorpholine buffer, pH 8.0, being taken as 0.029 M (Dawson, Elliott, Elliott and Jones, 1959). From this, values of V at different concentrations of Cl^- were calculated and are compared with the observed values in Table 17. It is seen that a decrease in maximum velocity with increasing

TABLE 17

The predicted effect of Cl^- as a non-competitive inhibitor, with respect to $MgADP^-$, of the reaction catalysed by creatine kinase

Cl^- is taken as having a K_i of 0.11 M (Nihei *et al.*, 1961). The calculated velocities are obtained from

$$V = \frac{V^*}{1 + \frac{i}{K_i}}$$

The calculated values, at PC = 5 mM, are compared with the observed values in Table 14, using the value of $V^* = 0.171$. All V values expressed in μmoles of creatine released per μg. of creatine kinase per min.

Mg^{2+} concentration (mM)	V (calculated)	V (observed)
1	0.133	0.128
5	0.126	0.103
10	0.118	0.078
15	0.111	0.067

Cl^- concentration would be predicted, though it was much less than the observed effect.

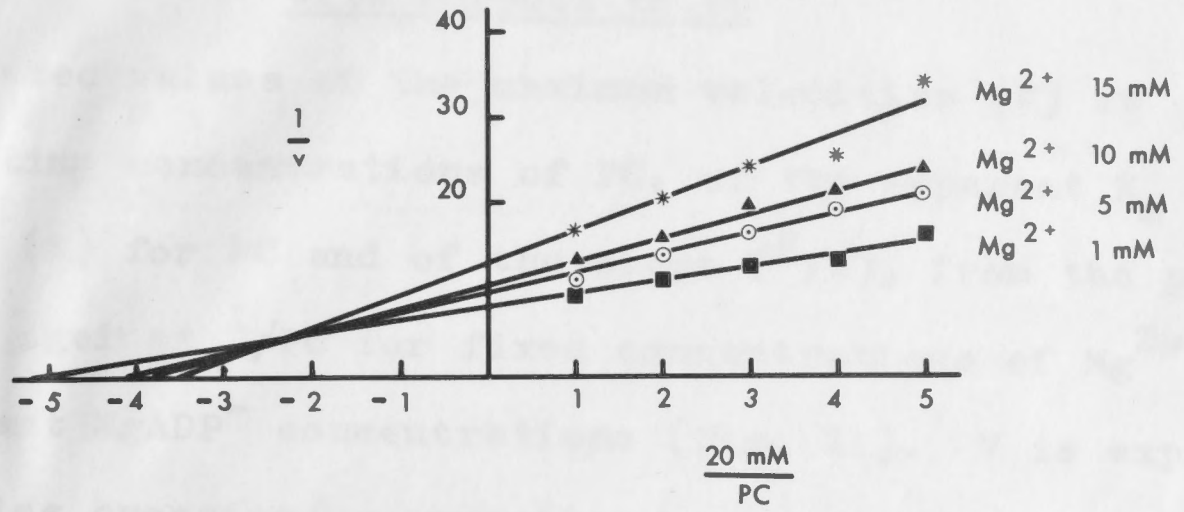
Conclusion: Each of the factors considered in this section appears to be unable to explain the observed inhibition. It is possible that the sum of the effects considered could make a substantial contribution to the total inhibition, but it was not possible to assess the possible magnitude of such a contribution.

The inhibition of creatine kinase by Mg^{2+} with respect to phosphorylcreatine (PC).

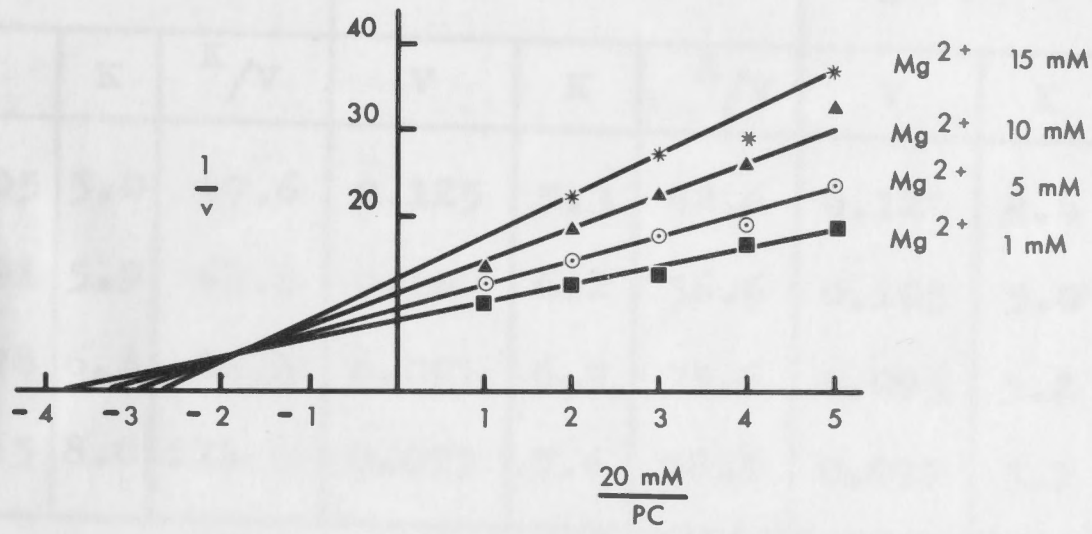
As the K_i values obtained from the inhibition of creatine kinase by Mg^{2+} with respect to $MgADP^-$ had been found to be dependent on the PC concentration, a more detailed analysis of the inhibition by Mg^{2+} with respect to PC was carried out. Reciprocal plots of the variation of velocity with PC concentration, obtained in the presence of different fixed concentrations of $MgADP^-$, are shown in Fig. 21. Both the slopes and the intercepts are functions of the Mg^{2+} concentration, so that the inhibition by Mg^{2+} is non-competitive. The values of the K_m for PC, the maximum velocities at saturating concentrations of PC, and of the slopes, calculated from these plots, are collected in Table 18. Plots of the slopes and of the reciprocals of the maximum velocities against Mg^{2+} concentration are shown in Fig. 22a and 22b. Both

Fig. 21. The inhibition by Mg^{2+} with respect to PC at three concentrations of $MgADP^-$. The concentrations of Mg^{2+} and $MgADP^-$ are indicated on the plots. The reaction mixtures contained N-ethylmorpholine (pH 8.0), 0.1 M; creatine kinase (0.576 µg.) and EDTA (10^{-5} M) as well as the indicated amounts of PC, Mg^{2+} and $MgADP^-$. Total volume, 1.0 ml., temp. 30°. Velocity is expressed as µmoles of creatine/µg. of creatine kinase/min.

Mg ADP$^-$ = 0.2 mM



Mg ADP$^-$ = 0.13 mM



Mg ADP$^-$ = 0.1 mM

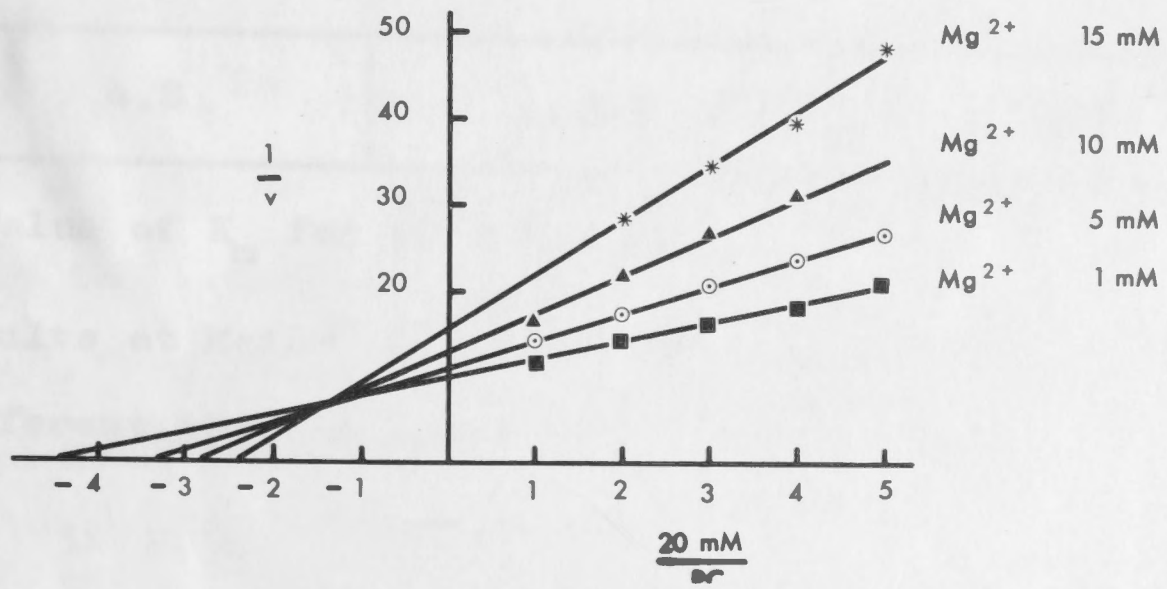


TABLE 18

The inhibition of creatine kinase by Mg^{2+} with respect to PC

Calculated values of the maximum velocities (V) at saturating concentrations of PC, of the apparent K_m values (K) for PC and of the slope ($^K/_V$), from the plots of $^1/_v$ against $^1/_{PC}$ for fixed concentrations of Mg^{2+} at different $MgADP^-$ concentrations (Fig. 21). V is expressed as μmoles creatine/μg. creatine kinase/min.; K as mM.

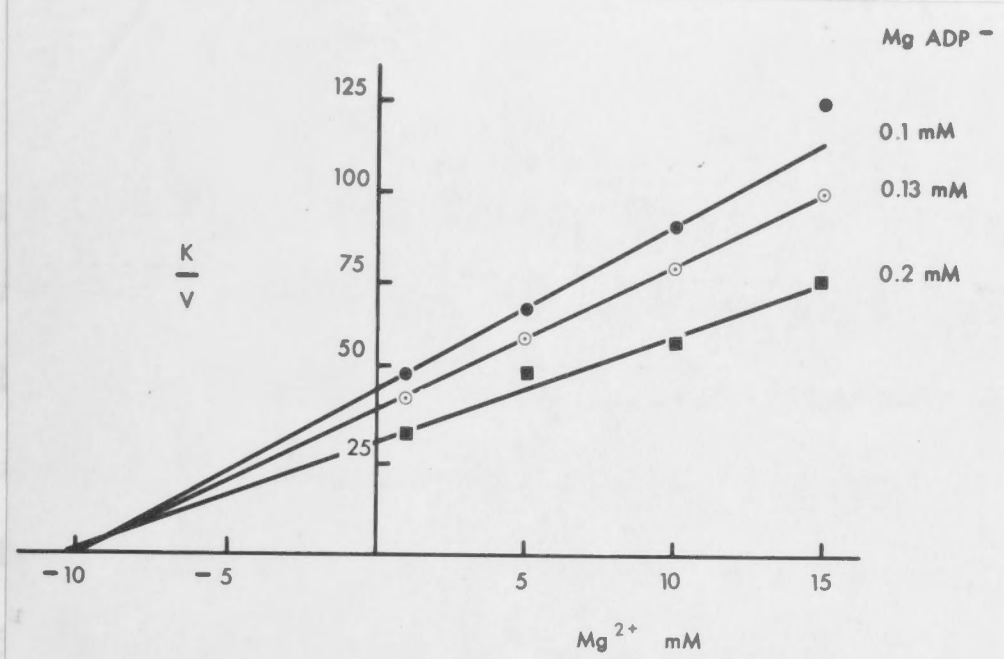
Mg^{2+} conc. (mM)	$MgADP^-$ = 0.1 mM			$MgADP^-$ = 0.13 mM			$MgADP^-$ = 0.2 mM		
	V	K	$^K/_V$	V	K	$^K/_V$	V	K	$^K/_V$
1	0.105	5.0	47.6	0.125	5.3	42.6	0.128	4.4	34.6
5	0.091	5.9	64.8	0.109	6.2	56.6	0.105	5.0	47.6
10	0.078	6.8	86.8	0.091	6.9	75.8	0.095	5.2	54.6
15	0.065	8.0	124	0.077	7.6	98.2	0.077	5.7	74.2

Values of K obtained by extrapolation to zero Mg^{2+} concentration:

	4.8	5.3	4.5

Average value of K_m for PC = 4.9 mM.

(N.B. Results at $MgADP^-$ = 0.2 mM were carried out at a different time to those at 0.13 and 0.1 mM.)



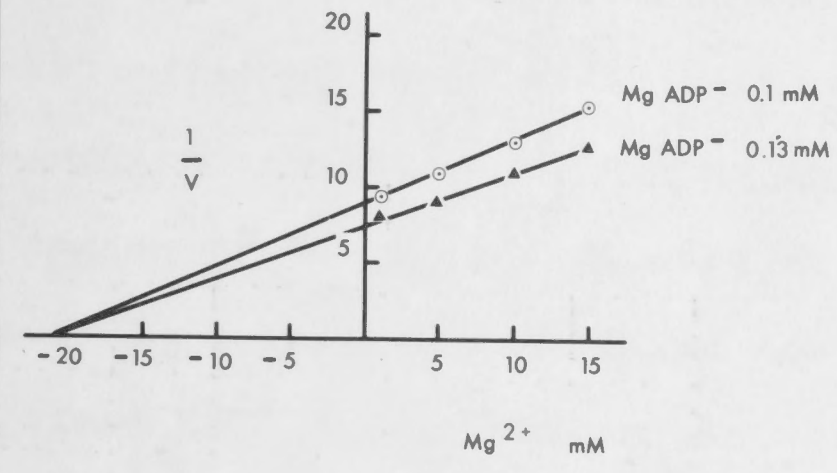


Fig. 22. (a) Plot of slope (K/V) against Mg^{2+} concentration at different $MgADP^-$ concentrations. Values calculated from the plots for the inhibition by Mg^{2+} with respect to PC (Fig.21).

(b) Plot of intercept (1/V) against Mg^{2+} at different $MgADP^-$ concentrations. Values calculated from the plots for the inhibition by Mg^{2+} with respect to PC (Fig. 21). (The results with $MgADP^-$ = 0.2 mM are not included in Fig. 22(b) as the experiments were carried out at a different time and the 1/V values fall almost exactly on the line for $MgADP^-$ = 0.13 mM. The lines of best fit were drawn using the method of least squares).

intercept and slope are linear functions of the Mg^{2+} concentration so that the inhibition can be more precisely classified as being linear non-competitive. The values of K_i (intercept) and K_i (slope) obtained from these plots are given in Table 19, and the values of 21 mM and 10.5 mM obtained are in good agreement with those of 20.3 mM at infinite PC concentration and of 12.8 mM at zero concentration of PC (Fig. 18). No effect of the $MgADP^-$ concentration on the K_i values was observed.

Estimates of the K_m for PC were obtained by plotting the apparent K_m values at each fixed concentration of $MgADP^-$ against Mg^{2+} concentration and extrapolating to zero (Fig. 23). These values are included in Table 18. In agreement with Nihei *et al.* (1961), though these workers obtained a much lower value, the K_m value for PC was independent of the $MgADP^-$ concentration in the range studied.

The possible effect of Cl^- on the apparent K_m for PC, using the values obtained at $MgADP^-$ = 0.1 mM, is shown in Table 20. The value, K^*, at zero concentration of Cl^-, was calculated from

$$K = K^* \left\{ 1 + \frac{i}{K_i} \right\}$$

with K = 4.8 mM and K_i = 0.11 M (Nihei *et al.*, 1961), to be 3.7 mM. It is seen that the effect is significant,

TABLE 19

Variation of K_i (Mg^{2+}) with $MgADP^-$ concentration

Estimates of K_i for Mg^{2+}, with respect to PC, at different fixed concentrations of $MgADP^-$. The values reported were obtained from the secondary plots of slope and intercept against Mg^{2+} concentration (Fig. 22a, 22b).

$MgADP^-$ (mM)	K_i (intercept) (mM)	K_i (slope) (mM)
0.1	21.1	9.9
0.13	21.0	9.5
0.2	21.1	12.0
Average:	21.1	10.5

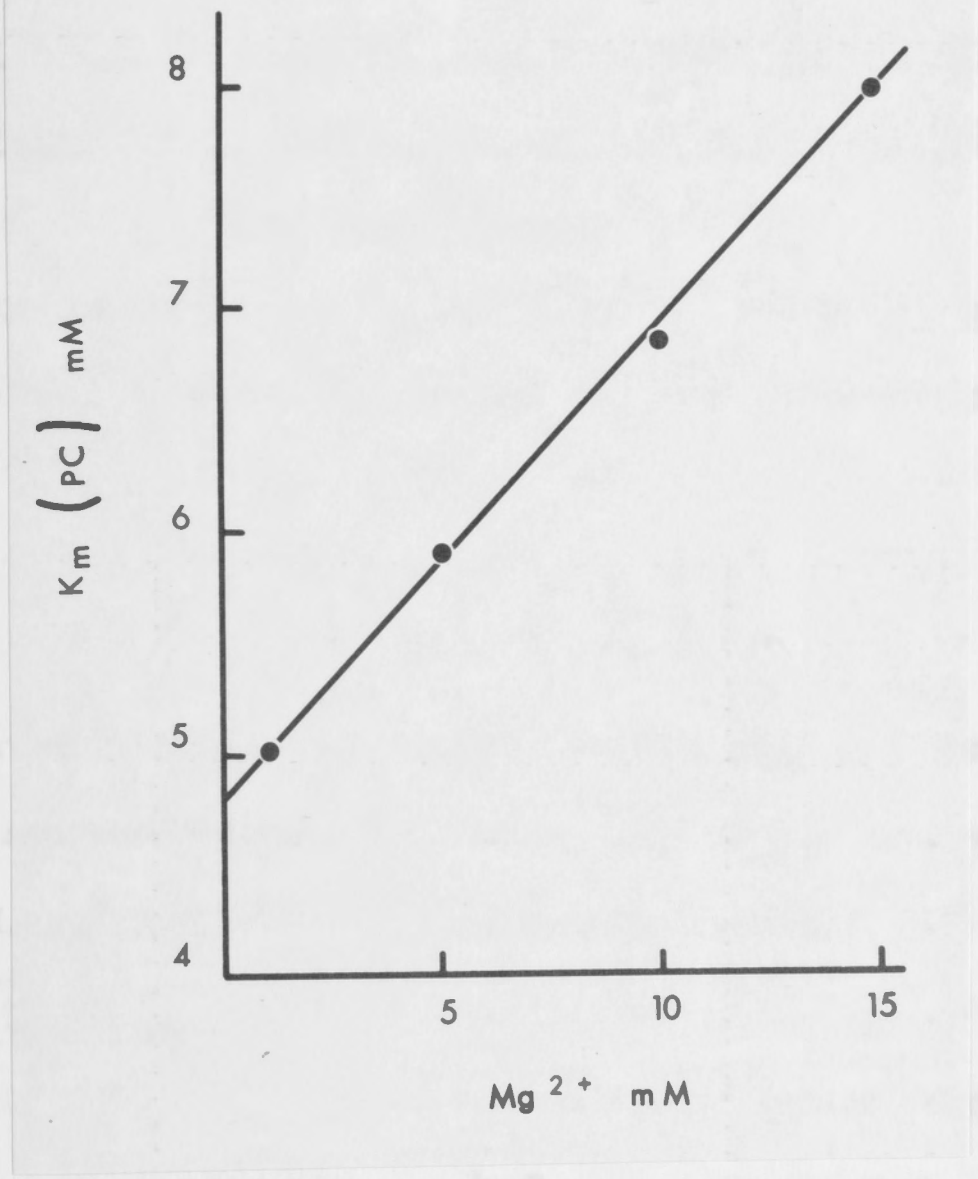


Fig. 23. Plot of apparent K_m for PC from the inhibition by Mg^{2+} with respect to PC (Fig. 21) at $MgADP^-$ = 0.1 mM, against Mg^{2+} concentration.

TABLE 20

The predicted effect of Cl^- as a competitive inhibitor, with respect to PC, of the reaction catalysed by creatine kinase

Cl^- is taken as having a K_i of 0.11 M (Nihei *et al.*, 1961). The calculated values of K_m for PC were obtained from

$$K = K^* \left\{ 1 + \frac{i}{K_i} \right\}$$

The calculated values, at $MgADP^-$ = 0.1 mM, are compared with the observed values in Table 18, using the value of K^* = 3.7 mM. All K values expressed as mM.

Mg^{2+} concentration (mM)	K_m (calculated)	K_m (observed)
1	4.9	5.0
5	5.1	5.9
10	5.5	6.8
15	5.8	8.0

though insufficient to explain the observed inhibition.

The possibility that the inhibition was to some extent due to the formation of an inactive MgPC complex was again considered, using the value of 12 M^{-1} for the stability constant of this complex. The effect on the plots of $^1/v$ against $^1/PC$ (Fig. 21) was to slightly reduce the apparent K_m for PC, while the maximum velocities remained unchanged. For instance, at $MgADP^-$ = 0.1 mM, with Mg^{2+} = 1 mM, the apparent K_m is unchanged, but at Mg^{2+} = 15 mM it is reduced from 8.0 to 7.3 mM. The plot of intercept against Mg^{2+} (Fig. 22a) was unchanged, and for the plots of slope against Mg^{2+} (Fig.22b) and of apparent K_m against Mg^{2+} (Fig. 23), the slopes were altered but the estimates of K_i (Mg^{2+}) and of K_m for PC were virtually unchanged. Thus the observed inhibition would not appear to be due to any large degree to the formation of MgPC. However, it is possible that the stability constant is somewhat greater than 12 M^{-1} (Smith and Alberty (1956) reported a value of 20 M^{-1}), in which case the contribution to the inhibition by the formation of MgPC would be increased, though the stability constant would need to be considerably higher to have any appreciable effect.

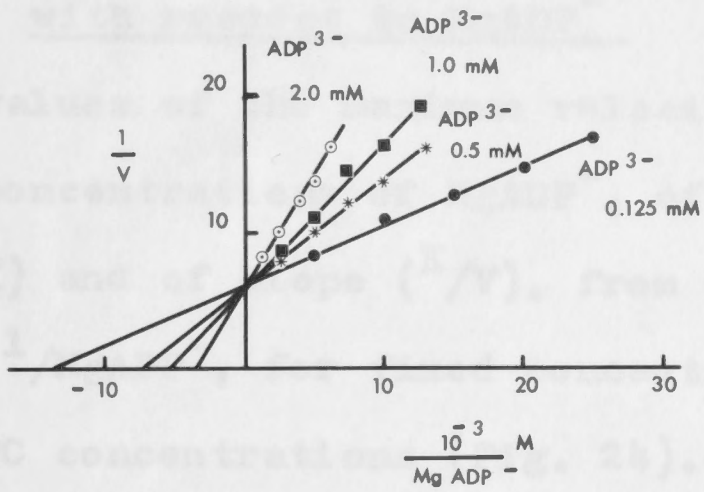
<u>The inhibition of creatine kinase by excess ADP^{3-} with respect to $MgADP^{-}$.</u>

The concentration of free ADP^{3-} was set at values in the range 0.125 to 2.5 mM and the concentration of $MgADP^{-}$ was varied from 1 to 0.033 mM. Under these conditions, the concentration of Mg^{2+} was negligible compared to K_i (Mg^{2+}). Plots of $^1/v$ against $^1/MgADP^{-}$ (Fig. 24) at three concentrations of PC, 5, 10 and 20 mM, show that ADP^{3-} inhibits the enzyme competitively with respect to $MgADP^{-}$. Calculated values, from these plots, of the K_m for $MgADP^{-}$, of the maximum velocity at saturating concentrations of $MgADP^{-}$ for each concentration of ADP^{3-}, and of the slope, are collected in Table 21. Secondary plots of slope against ADP^{3-} concentration are shown in Fig. 25 and the K_i values obtained from these plots in Table 22. As the slope is a linear function of the ADP^{3-} concentration, the inhibition may be classified as linear competitive.

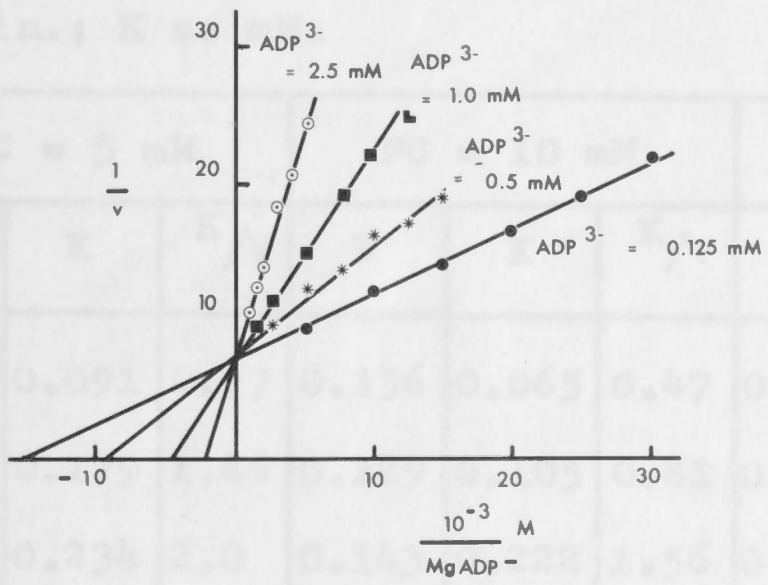
This data may also be treated in terms of the general equation. From equation (9), derived from the general velocity equation for the condition that $\sigma \gg K_4$, plots of $^1/v$ against $^1/MgADP^{-}$, for fixed values of ADP^{3-} (σ'), should give a series of straight lines with the same intercept, as in Fig. 24, and with $K_{\sigma'} = K_6(1 + \frac{\sigma'}{K_1})$ (Equation 10). (It should be mentioned that, if the values of K_4 reported in Chapter IV, obtained using the average

Fig. 24. The inhibition of creatine kinase by ADP^{3-} with respect to $MgADP^{-}$ at different PC concentrations. The concentrations of Mg^{2+} and $MgADP^{-}$ were adjusted by varying the total concentrations of ADP and Mg, with values of 3,300 M^{-1}, 3,600 M^{-1} and 3,800 M^{-1} for the stability constant of $MgADP^{-}$ at PC equal to 20, 10 and 5 mM respectively. The reaction mixtures contained N-ethylmorpholine (pH 8.0), 0.1 M; creatine kinase, 0.576 µg. and EDTA as well as the indicated amounts of ADP^{3-}, PC and $MgADP^{-}$. Total volume, 1.0 ml., temp. 30°. Velocity is expressed as µmoles of creatine/µg. of creatine kinase/min. (Only four plots are shown at PC = 10 mM as otherwise the graph became too congested).

PC = 20 mM



PC = 10 mM



PC = 5 mM

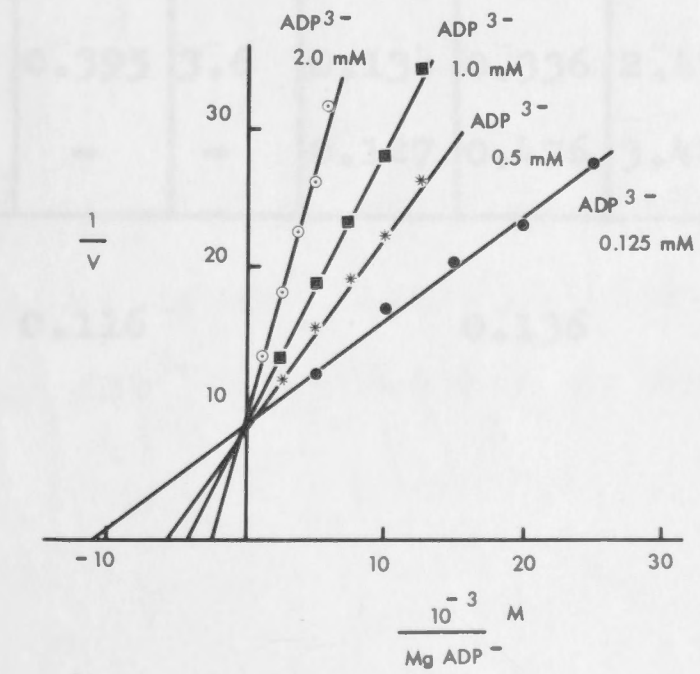


TABLE 21

The inhibition of creatine kinase by ADP^{3-} with respect to $MgADP^-$

Calculated values of the maximum velocities (V) at saturating concentrations of $MgADP^-$, of the apparent K_m values (K) and of slope ($^K/_V$), from the plots of $^1/_v$ against $^1/_{MgADP^-}$, for fixed concentrations of ADP^{3-}, at various PC concentrations (Fig. 24). V is expressed as μmoles of creatine released per μg. of creatine kinase per min.; K as mM.

ADP^{3-} (mM)	PC = 5 mM			PC = 10 mM			PC = 20 mM		
	V	K	$^K/_V$	V	K	$^K/_V$	V	K	$^K/_V$
0.125	0.118	0.091	0.77	0.136	0.065	0.47	0.163	0.072	0.44
0.5	0.121	0.179	1.46	0.129	0.105	0.81	0.170	0.135	0.80
1.0	0.116	0.234	2.0	0.143	0.222	1.56	0.170	0.187	1.09
1.5	-	-	-	0.145	0.308	2.16	-	-	-
2.0	0.110	0.395	3.6	0.134	0.336	2.47	0.164	0.258	1.57
2.5	-	-	-	0.127	0.476	3.48	-	-	-
Average V	0.116			0.136			0.167		

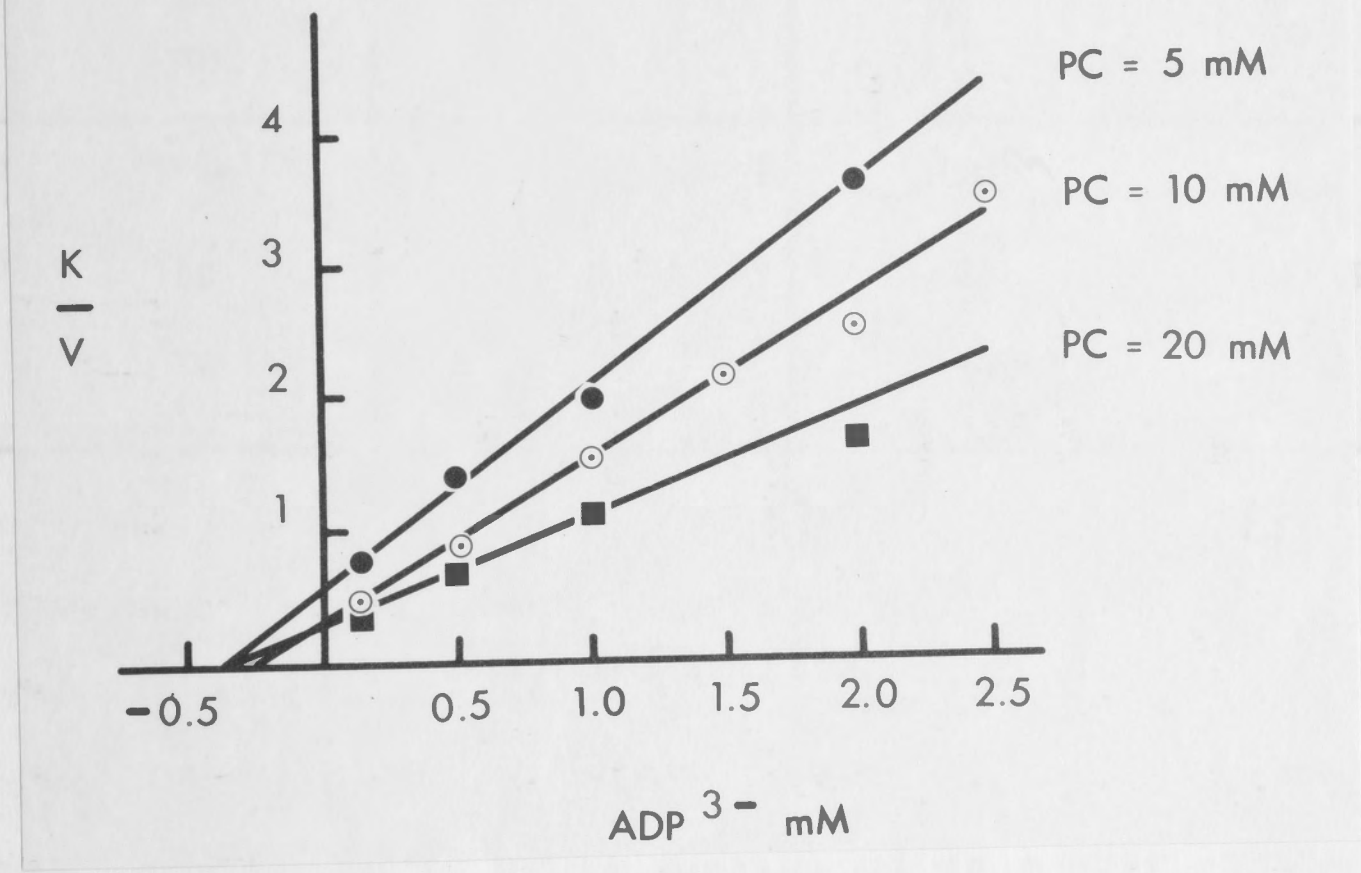


Fig. 25. Plots of slope (K/V) against ADP^{3-} concentration using the values obtained from the inhibition by ADP^{3-} with respect to $MgADP^{-}$ (Fig.24).

TABLE 22

Values of the K_i for ADP^{3-} as a competitive inhibitor of $MgADP^-$, and of the constants K_1 and K_6 as obtained from the plots in Fig. 25 (K_i) and Fig. 26 (K_1 and K_6). All K values M x 10^4.

PC (mM)	K_i	K_1	K_6
5	4	5	0.8
10	2	2	0.35
20	4	4.5	0.6

K_μ, values are correct, then the condition, $\sigma' \gg K_4$, does not hold for the lower concentrations of σ' used in this study. However, it would be valid for the much lower values of K_4 reported in Chapter VI.) In Fig. 26, $K_{\sigma'}$ has been plotted against σ', for the $K_{\sigma'}$ values obtained at PC = 10 mM. This plot gave values of K_1, the constant for the interaction between ADP^{3-} and the enzyme, and of K_6, the constant for the interaction between $MgADP^-$ and the enzyme, from the intercepts on the x- and y-axis respectively.

The values of K_1 and K_6 obtained from this plot, together with those for PC at 5 and 20 mM, are included in Table 22. If K_1 is a true dissociation constant, then it should be identical to K_i. This is supported by the figures reported in Table 22, i.e., the value for the interaction of ADP^{3-} with the enzyme is the same, whether this interaction is being considered as a step in the reaction pathway or as inhibiting the reaction. It should be noted that the values for K_1, especially with PC at concentrations of 5 and 20 mM, are somewhat higher than those reported in Chapter IV. This could be due to experimental error, as only four concentrations of ADP^{3-} were considered in these two cases, and a small change in the apparent K_m for ADP^{3-} could have a marked effect on the plot of $K_{\sigma'}$ against σ'. By contrast, the values for

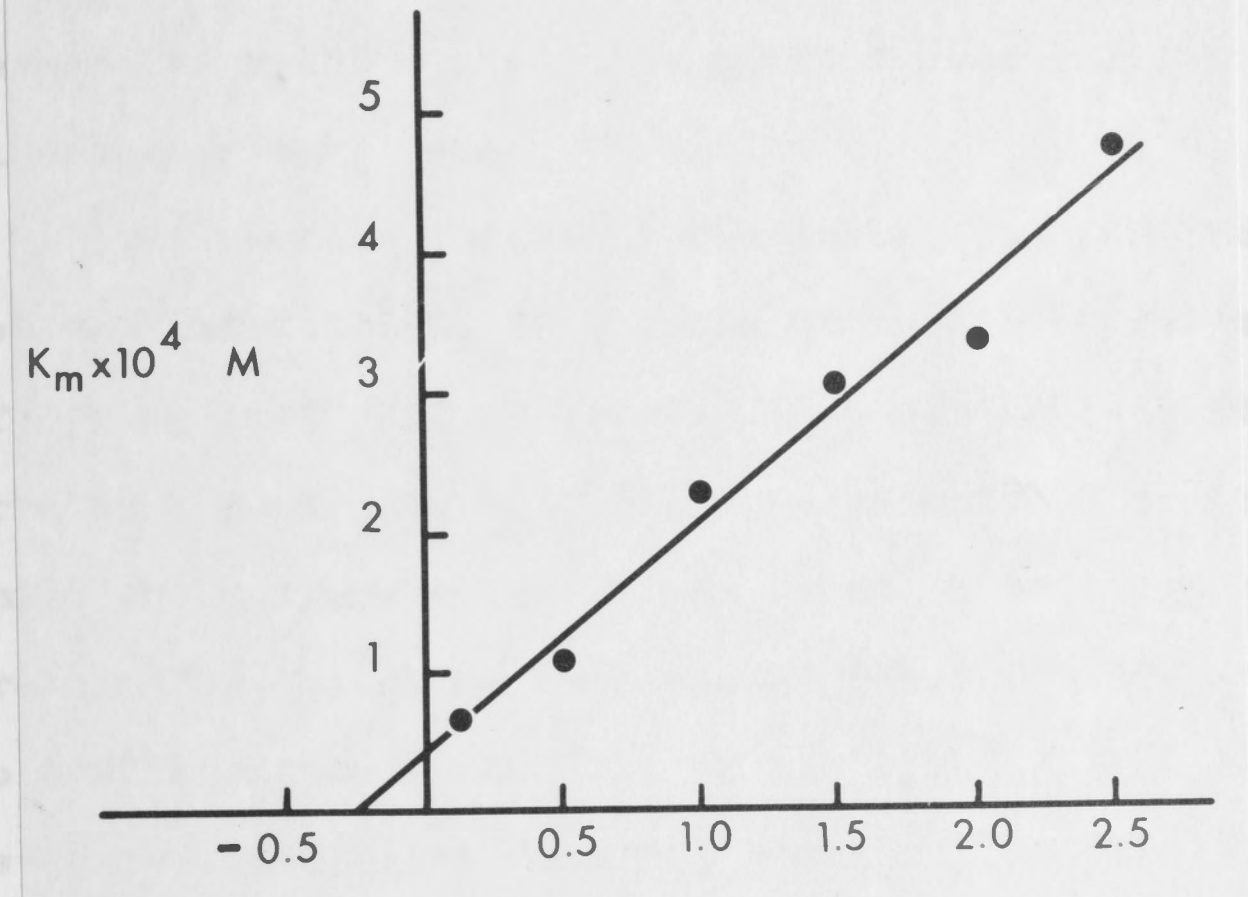


Fig. 26. Plot of K values, calculated from the data for the inhibition of creatine kinase by ADP^{3-} with respect to $MgADP^-$, at PC = 10 mM (Fig. 24).

K_6 were somewhat lower than those reported in Chapter IV, and to a lesser degree, lower than the value obtained for the K_m for $MgADP^-$ from the inhibition by Mg^{2+} with respect to $MgADP^-$, though in neither case was the discrepancy very great.

The average values of the intercept, for each concentration of PC, from Table 21 were used for a plot of $^1/V$ against $^1/PC$, according to equation (4a) (Fig. 27). From this plot, the K_m for PC was calculated to be 3.6 mM, while the maximum velocity was found to be 0.193 μmoles creatine/μg. creatine kinase/min. (15,600 moles/min./ mole of creatine kinase) at pH 8.0 with the enzyme saturated with $MgADP^-$ and PC, and ADP^{3-} at zero concentration. This may be compared with the value of 0.23 μmoles creatine/μg. creatine kinase/min. obtained under identical conditions with the Mg^{2+} concentration extrapolated to zero.

<u>The inhibition of creatine kinase by excess ADP^{3-} with respect to phosphorylcreatine</u>.

If ADP^{3-} reacts primarily at a different site on the enzyme to that at which PC reacts, it would be expected that the inhibition by excess ADP^{3-}, with respect to PC, would be of the simple, non-competitive type. However, it was found that the inhibition did not have a simple character. Plots of $^1/v$ against $^1/PC$, at $MgADP^-$

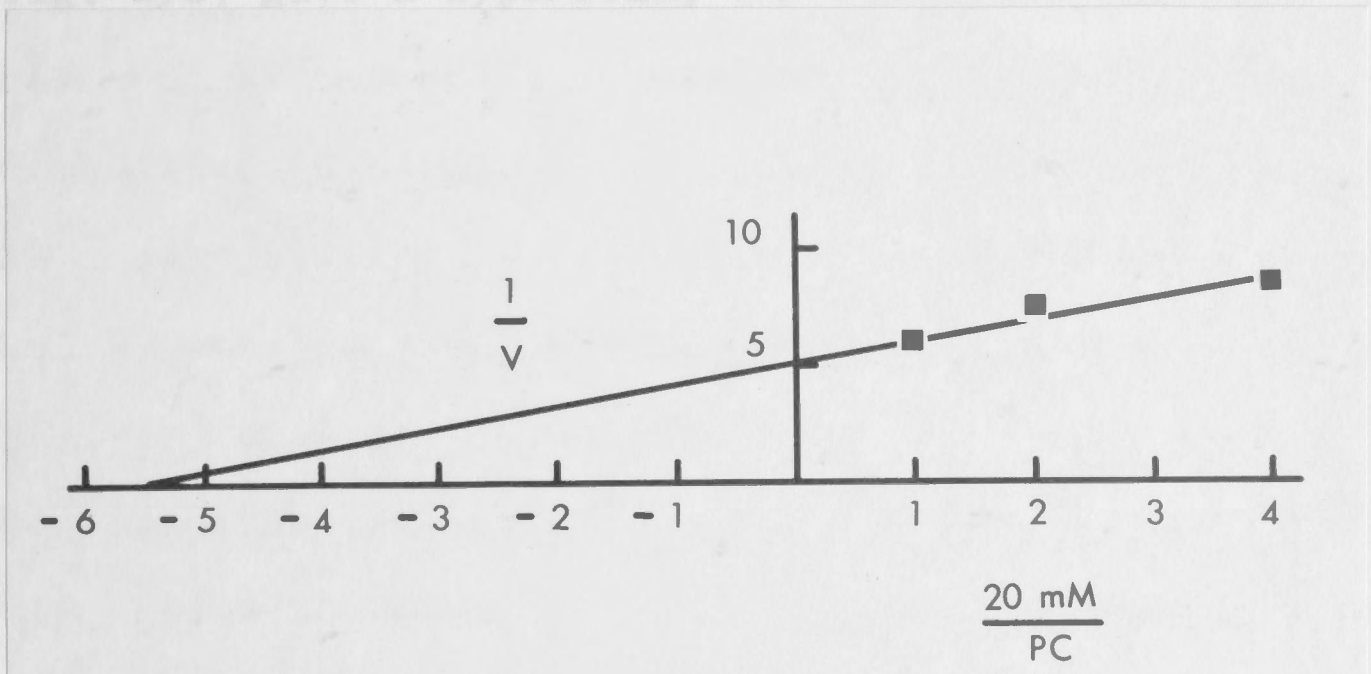


Fig. 27. Plot of 1/V against 1/PC, using the average V values calculated from the plots in Fig. 24, for the inhibition of creatine kinase by ADP^{3-} with respect to $MgADP^{-}$.

equal to 0.2 mM, for different values of ADP^{3-} are shown in Fig. 28, and the calculated values for the K_m for PC, the maximum velocity at saturating levels of PC at each concentration of ADP^{3-}, and for the slope are collected in Table 23. Because a plot of slope against ADP^{3-} was linear (Fig. 29a) while a plot of intercept against ADP^{3-} (Fig. 29b) gave a hyperbola, the inhibition may be described as intercept-hyperbolic slope-linear non-competitive (Cleland, 1963). The plot of slope against ADP^{3-} gave a value of 0.75 mM for the K_i of ADP^{3-}. For the hyperbolic plot, the K_i may be obtained by plotting $1/(V_i-V')$ against $1/i$, where V' is the value of V when the inhibitor concentration, i, is zero (Cleland, 1963). Such a plot is shown in Fig. 29c, using a value of V' = 0.20 μmoles creatine/μg. of creatine kinase/min., obtained from Fig. 29b. The K_i for ADP^{3-}, determined from this plot, was 0.55 mM. (In calculating K_i, the point corresponding to ADP^{3-} = 0.125 mM was neglected. This point fell a long way from the line drawn and would be subject to the greatest experimental error, as it is the most sensitive to the value of V'.)

A value for the K_m for PC was obtained by plotting apparent K_m values, for each concentration of ADP^{3-}, against ADP^{3-} concentration (Fig. 30) and extrapolating the parabolic curve shown to zero concentration of ADP^{3-} to give the value of 4.5 mM.

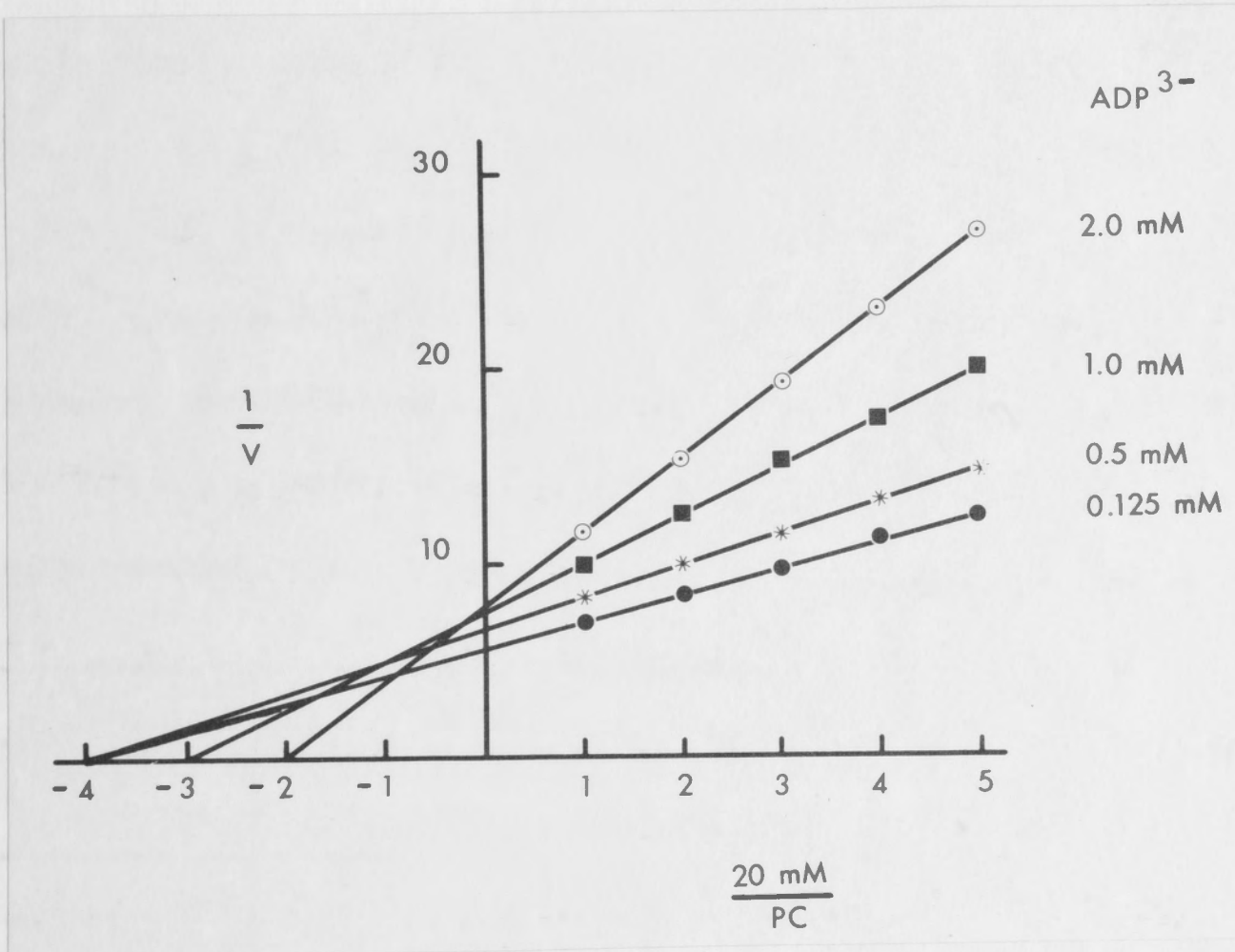


Fig. 28. The inhibition of creatine kinase by ADP^{3-} with respect to PC at $MgADP^{-}$ = 0.2 mM. The reaction mixtures contained N-ethylmorpholine (pH 8.0), 0.1 M; creatine kinase, 0.576 µg. and EDTA (5×10^{-6} M) as well as the indicated amounts of ADP^{3-}, PC and $MgADP^{-}$. Total volume, 1.0 ml., temp. 30°. Velocity is expressed as µmoles of creatine/µg. of creatine kinase/min.

TABLE 23

The inhibition of creatine kinase by ADP^{3-} with respect to PC

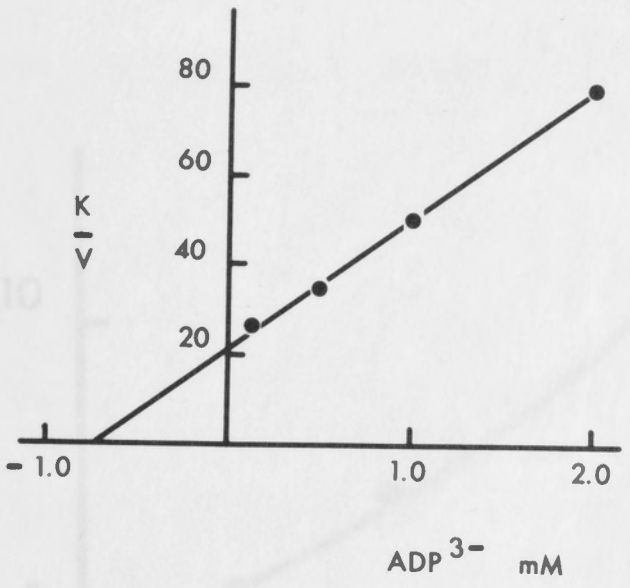
Calculated values of the maximum velocities (V) at saturating concentrations of PC, of the apparent K_m values (K) for PC and of the slope ($^K/V$), from the plots of $^1/v$ against $^1/PC$, for fixed concentrations of ADP^{3-}, with $MgADP^-$ fixed at 0.2 mM. V is expressed as μmoles creatine/μg. creatine kinase/min.; K as mM. Figures in parenthesis are results from another experiment.

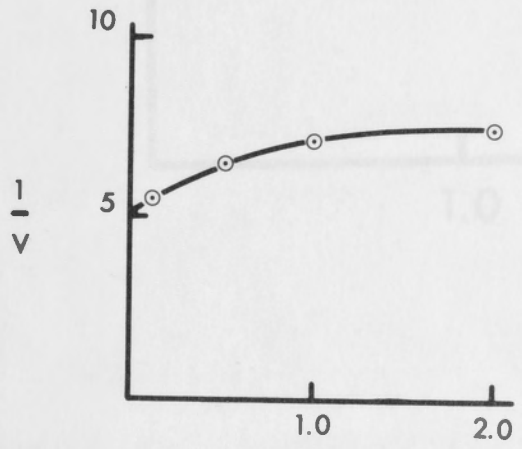
ADP^{3-} (mM)	V	K	$^K/V$
0.125	0.181	4.8 (5.1)	26.5
0.5	0.156	5.4 (5.4)	34.6
1.0	0.141	7.2 (7.3)	51.1
2.0	0.135	10.8 (13.6)	80.0

Fig. 29 (a). Plot of slope (K/V) against ADP^{3-} using the values calculated from the plots for the inhibition by ADP^{3-} with respect to PC (Fig. 28).

(b). Plot of intercept (1/V) against ADP^{3-} using the values calculated from the plots for the inhibition by ADP^{3-} with respect to PC (Fig. 28).

(c). Plot of $^{1}/(V_i - V^1)$ against 1/i, where i = ADP^{3-} concentration (see text).


80
60
K/V
40
20
- 1.0
1.0
2.0
ADP 3- mM


10
1/V
5
1.0
2.0
ADP 3- mM

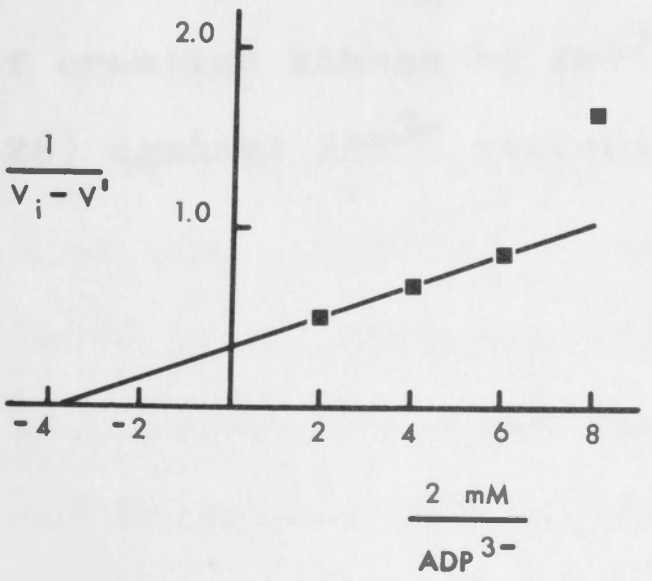

2.0
1/(Vi - V')
1.0
-4
-2
2
4
6
8
2 mM / ADP 3-

TABLE 24

Summary of the character of the inhibitions by excess Mg^{2+} and excess ADP^{3-} with respect to $MgADP^{-}$ and PC

The nomenclature used to describe the type of inhibition is that suggested by Cleland (1963). $K_{i(1)} = K_i$ (intercept); $K_{i(2)} = K_i$ (slope). All K values expressed as mM.

Inhibitor	Substrate	$K_{i(1)}$	$K_{i(2)}$	Type of inhibition	Form of rate equation descriptive of the inhibition
Mg^{2+}	$MgADP^{-}$	12.8	12.8	Simple linear non-competitive	$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{i}{K_{i(1)}}\right)\frac{1}{S} + \frac{1}{V}\left(1 + \frac{i}{K_{i(2)}}\right)$ with $K_{i(1)} = K_{i(2)}$
Mg^{2+}	PC	21.1	10.5	Linear non-competitive	$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{i}{K_{i(1)}}\right)\frac{1}{S} + \frac{1}{V}\left(1 + \frac{i}{K_{i(2)}}\right)$
ADP^{3-}	$MgADP^{-}$	0.3	-	Linear competitive	$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{i}{K_i}\right)\frac{1}{S} + \frac{1}{V}$
ADP^{3-}	PC	0.75	0.55	Mixed (slope-linear; intercept-hyperbolic) non-competitive inhibition	$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{i}{K_{i(1)}}\right)\frac{1}{S} + \frac{1}{V}\left\{\frac{a + \frac{bi}{K_{i(2)}}}{c + d\frac{i}{K_{i(2)}}}\right\}$ where $bc > ad$

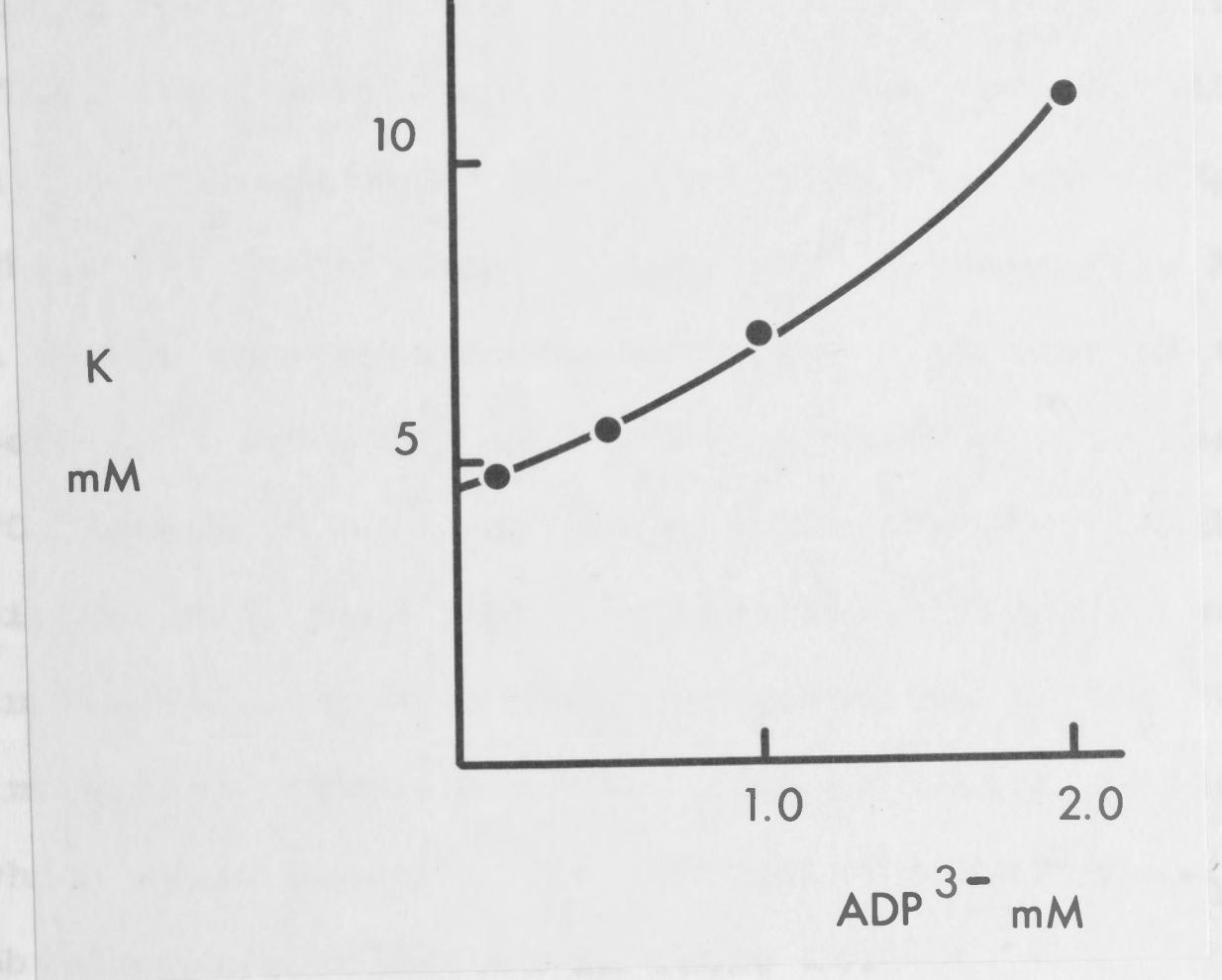


Fig. 30. Plot of apparent K_m for PC from the inhibition of creatine kinase by ADP^{3-} with respect to PC (Fig. 28) against ADP^{3-} concentration.

TABLE 25

The interaction of creatine kinase with its reaction components

A comparison between the kinetically determined values reported in this chapter and the thermodynamically determined values obtained by Kuby, Mahowald and Noltmann (1962). All values expressed as M.

Component	$K_{(kinetic)}$	$K_{eq.}$
ADP^{3-}	$2 - 5 \times 10^{-4}$ (2.7×10^{-4})*	1×10^{-4}
$MgADP^{-}$	$3.5 - 10 \times 10^{-5}$ (5×10^{-5})	$6 - 7 \times 10^{-5}$
Mg^{2+}	$1 - 2 \times 10^{-2}$	pK = 2
PC	$3.6 - 5 \times 10^{-3}$	pK = 2 to 3

*Values in parenthesis are estimates obtained from the kinetic studies reported in Chapter VI.

The inhibition by ADP^{3-} with respect to PC was more complex and no explanation was readily forthcoming, on the basis of equilibrium kinetics, to explain the hyperbolic nature of the plot of intercept against ADP^{3-} concentration (Fig. 29b).

The K_i values obtained for Mg^{2+}, as a non-competitive inhibitor with respect to both $MgADP^-$ and PC, were greater by two orders of magnitude than the values reported in Chapter IV for K_3, the dissociation constant for the interaction between Mg^{2+} and the enzyme. Further, it was not possible to reconcile the results with the initial velocity equation expressed in terms of Mg^{2+} and $MgADP^-$. Thus, the results are inconsistent in part with those reported in Chapter IV, insofar as the inhibition experiments indicate that Pathway II ($E + M \overset{K_3}{\rightleftharpoons} EM \overset{K_4}{\rightleftharpoons} EMS$) does not operate in the formation of the active EMS complex.

As the K_i for Mg^{2+} was dependent on the PC concentration, though not on the $MgADP^-$ concentration, it was possible to obtain values graphically (Fig. 18) for the K_i at zero and at infinite concentration of PC. Thus the inhibition constant observed under any particular set of conditions would lie somewhere between $K_{i'} = 12.8$ mM, the value when the PC concentration is zero, and $K_{i''} =$

22 mM, when the enzyme is saturated with PC. These results would also indicate that Mg^{2+} is acting at two sites on the enzyme, one independent to the active site and the other such that it influences the binding of PC to the enzyme. The latter could be due to competition between Mg^{2+} and the creatine moiety of PC for a negative site on the enzyme. (N.B. The variation in the apparent K_m for PC is contrary to what was reported in Chapter IV, but in the latter case the Mg^{2+} range studied was too narrow to have observed any significant variation).

Factors that could have produced an apparent non-competitive inhibition by excess Mg^{2+} were the formation of complexes such as Mg_2ADP^+ and MgPC, increased ionic strength and inhibition by chloride ion, as Mg^{2+} was added as $MgCl_2$. While each of these effects was shown to be unable singly to account for the inhibition, it is possible that their cumulative effect could have given rise to the experimental observations. However, the similarity of the K_i values for Mg^{2+} to the thermodynamic values reported by Kuby et al. (1962) (see Table 25) appeared to indicate that there was a direct interaction with the enzyme, though not necessarily at the active site. It is interesting to note that the studies of Kuby et al. (1962) indicated that Mg^{2+} was bound to four sites on creatine kinase, though the enzyme appeared to

have only two active sites.

It is of interest to note that the PC concentration had little, if any, effect on the K_m value obtained for $MgADP^-$ (Table 14). This would validate the original assumption (Chapter IV) that the concentration of PC altered the velocity by a constant factor. It is also indicative of the reaction being random, which type of reaction has only been previously demonstrated for pyruvate kinase (Reynard et al., 1961).

The kinetically determined results, from this chapter, for the interaction of creatine kinase with its reaction components are compared with the thermodynamic values of Kuby et al. (1962) in Table 25. From the generally good agreement between the kinetic and thermodynamic values, the assumption that the enzymic reaction obeys equilibrium kinetics appears to be tenable. Kinetic values for ADP^{3-} and $MgADP^-$ obtained from Chapter VI have also been included in Table 25.

It is worth drawing particular attention to two results recorded in this table. Firstly, the K_m for PC, for which values between 3.6 and 5 mM were consistently obtained: previously reported values have ranged from 0.8 mM to 16 mM (Kuby et al., 1954b; Nihei et al., 1961; Morrison et al., 1961). Secondly, that $MgADP^-$ binds more strongly to the enzyme than free ADP^{3-}; i.e., even if Mg^{2+}

does not itself bind to the enzyme, it still, in effect, facilitates the binding of ADP.

The inhibition studies were initiated to confirm the conclusions drawn in Chapter IV. As pointed out above, they are not in full agreement with these conclusions insofar as the inhibition experiments indicated that Pathway II, via the species, EM, was not operative in the formation of the active EMS complex. The original kinetic study should have been sufficient to eliminate any non-operative pathways and it seemed possible that some error occurred in this work. Hence it was decided to repeat the experiments for the reverse reaction taking into account the value for the stability constant of $MgADP^-$, under different conditions, as reported in Chapter II. This is described in the next chapter.

SUMMARY

The inhibition experiments showed that ADP^{3-} was a competitive inhibitor, and Mg^{2+} a non-competitive inhibitor, with respect to $MgADP^-$, for the reaction catalysed by creatine kinase. Thus it appeared unlikely that EM functioned as an intermediate in the formation of the EMS complex.

The experiments also indicated that the reaction was random and obeyed equilibrium kinetics.

CHAPTER VI

A REASSESSMENT OF THE KINETICS OF THE REVERSE REACTION CATALYSED BY CREATINE KINASE

CHAPTER VI

A REASSESSMENT OF THE KINETICS OF THE REVERSE REACTION CATALYSED BY CREATINE KINASE

INTRODUCTION

The inhibition experiments described in the previous chapter were consistent in part with the conclusions drawn from Chapter IV, in that they indicated that ADP^{3-} reacts at an active centre of the enzyme. However, they also indicated that Mg^{2+} did not react at the active centre, this being contrary to the findings from Chapter IV. Thus, it was decided to carry out a reinvestigation of the kinetics of the reverse reaction catalysed by creatine kinase.

This chapter describes the results of the reinvestigation of the kinetics of the reverse reaction. In particular, the effect of PC concentration on the values of the various kinetic constants has been determined and for this purpose the values for the apparent stability constant of $MgADP^{-}$, in the presence of PC, as reported in Chapter II, have been used. Furthermore, as the results in Chapter V were obtained with reaction mixtures which contained EDTA, this reagent being absent from the experiments described in Chapter IV, experiments were carried out with and without EDTA, to see if this affected the results.

THEORY

The general velocity equation, in terms of $\sigma(ADP^{3-})$ and $\mu(Mg^{2+})$, may be written as

$$v = \frac{ke}{\frac{K_1K_2}{\sigma\mu} + \frac{K_2}{\mu} + \frac{K_4}{\sigma} + 1} \qquad (1)$$

Allowing for the interaction of PC with the enzyme

$$v = \frac{ke}{\left\{1 + \frac{K_7}{[PC]}\right\}\left\{\frac{K_1K_2}{\sigma\mu} + \frac{K_2}{\mu} + \frac{K_4}{\sigma} + 1\right\}} \qquad (2)$$

where K_7 is the equilibrium constant for the reaction

$$E + PC \rightleftharpoons E - PC$$

In the inverse form

$$\frac{1}{v'} = \frac{K_2}{ke} \cdot \frac{1}{\mu}\left\{\frac{K_1}{\sigma} + 1\right\} + \frac{1}{ke}\left\{\frac{K_4}{\sigma} + 1\right\} \qquad (3)$$

where:

$$\frac{1}{v} = \frac{1}{v'}\left\{1 + \frac{K_7}{[PC]}\right\} \qquad (4)$$

Thus a plot of $1/v'$ against $1/\mu$ should be linear, for σ held constant at σ', with intercepts at $1/V_{\sigma'}$ and $1/K_{\sigma'}$, where

$$V_{\sigma'} = \frac{ke}{\frac{K_4}{\sigma'} + 1} \qquad (5)$$

and

$$K_{\sigma'} = \frac{\frac{K_1K_2}{\sigma'} + K_2}{\frac{K_4}{\sigma'} + 1} \qquad (6)$$

Rearranging equation (5) gives

$$\frac{1}{V_{\sigma'}} = \frac{1}{\sigma'} \cdot \frac{K_4}{ke} + \frac{1}{ke} \qquad (7)$$

so that a plot of $^1/V_{\sigma'}$ against $^1/\sigma'$ gives a straight line with intercept $- {}^1/K_4$ on the x-axis.

Rearranging equation (6) gives

$$K_{\sigma'} \left(\frac{K_4}{\sigma'} + 1\right) = \frac{K_1 K_2}{\sigma'} + K_2 \qquad (8)$$

so that a plot of $K_{\sigma'} (\, {}^{K_4}/\sigma' + 1)$ against $^1/\sigma'$ gives intercepts K_2 and $- {}^1/K_1$ on the y- and x-axis respectively. Thus all the constants can be calculated from the relationship,

$$K_1 K_2 = K_3 K_4 = K_5 K_6,$$

K_5 having been previously determined.

If Mg^{2+} does not react with the enzyme, then $K_3 = \infty$, $K_4 = 0$, and equation (3) may be expressed as

$$\frac{1}{v'} = \frac{K_5 K_6}{ke} \; \frac{1}{\mu} \left\{ \frac{1}{\sigma} + \frac{1}{K_1} \right\} + \frac{1}{ke} \qquad (9)$$

Plots of $^1/v'$ against $^1/\mu$, for various fixed values of σ, should give a family of straight lines, intersecting at the same point, V, on the y-axis and at $- {}^1/K_\sigma$ on the x-axis, where

$$K_\sigma = K_5 K_6 \left\{ \frac{1}{\sigma} + \frac{1}{K_1} \right\} \qquad (10)$$

Then a plot of K_σ against $^1/\sigma$ should give a straight line

cutting the y- and x-axis at K_5K_6/K_1 and $-1/K_1$ respectively. Thus all the constants can be calculated from

$$K_1K_2 = K_5K_6 .$$

MATERIALS AND METHODS

A solution of PC was stirred with Chelex 100 (200-400 mesh, Na^+ form) resin to remove trace metals, the resin filtered off and the PC recrystallised from 80% ethanol. ADP (2×10^{-2} M) was also treated with Chelex and the resultant solution standardised at 259 mμ.

All enzymic assays were carried out in the presence of 5×10^{-6} M EDTA, unless indicated otherwise. This level of EDTA would have a negligible effect on the concentration of free Mg^{2+}. Otherwise the experimental conditions were as described in Chapter IV.

The amounts of Mg and ADP to be added to give the required concentrations of free Mg^{2+} and free ADP^{3-} were calculated using the values of 3,300, 3,600 and 3,800 M^{-1} (K_5 = 0.30, 0.28 and 0.265 mM) for the stability constant of $MgADP^-$ at PC concentrations of 20, 10 and 5 mM respectively. All reactions were carried out at two time periods (usually $\frac{3}{4}$ and $1\frac{1}{2}$ minutes) to ensure that initial velocities were being measured.

The lines of best fit for the double reciprocal

plots were drawn using values of maximum velocity and apparent K_m calculated by Wilkinson's (1961) method.

RESULTS

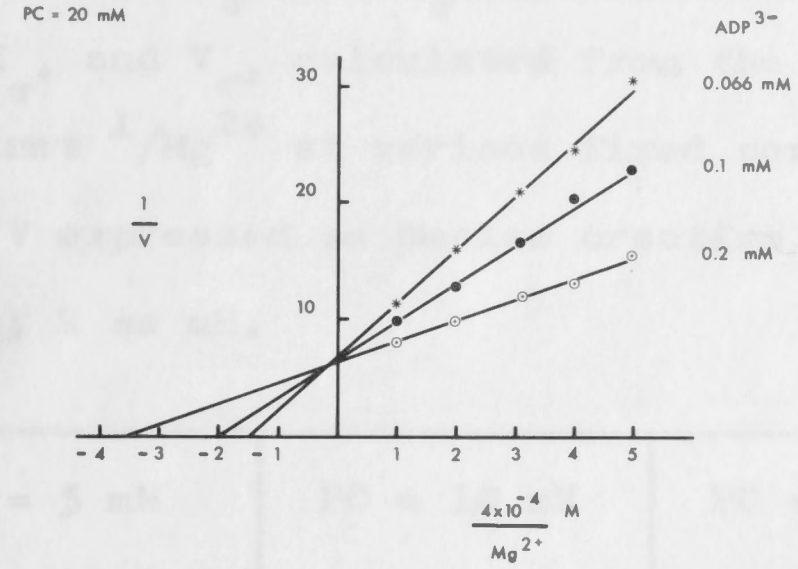
The activation of creatine kinase by Mg^{2+} in relation to ADP^{3-}.

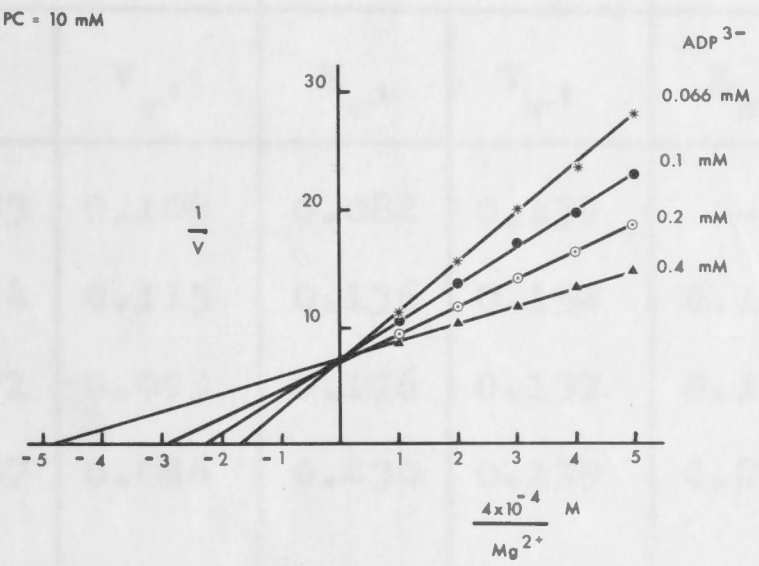
Plots of $^1/v$ against $^1/Mg^{2+}$, corresponding to equation (3), at various fixed concentrations of ADP^{3-}, are shown in Fig. 31 for three concentrations of PC. The calculated values for the maximum velocity (V_σ') and apparent K_m for Mg^{2+} (K_σ') at each concentration of ADP^{3-}, are collected in Table 26.

It is seen that there is a definite trend in the K_σ' values, K_σ' increasing with decreasing ADP^{3-} concentration, but that the V_σ' values cut either on or near the same point on the y-axis. Thus the results appear to fit the conditions for equation (9), implying that Mg^{2+} does not react with the enzyme.

In Fig. 32, K_σ' has been plotted against $^1/\sigma'$, corresponding to equation (10). This gave values for K_1 and for K_5K_6/K_1. Thus, using the relationship $K_1K_2 = K_5K_6$, and the value for K_5, the constants K_2 and K_6 could be calculated. These values are collected in Table 27. The constants appear to follow a trend, K_1 increasing and K_2 decreasing with increasing PC concentration. However, it is doubtful if this is

Fig. 31. Effect of the concentration of Mg^{2+} on the initial velocity of the reverse reaction catalysed by creatine kinase, at various fixed concentrations of ADP^{3-}, for different concentrations of PC. The concentrations of Mg^{2+} and ADP^{3-} were adjusted by varying the total concentrations of ADP and Mg^{2+}, with values of 3,300 M^{-1}, 3,600 M^{-1} and 3,800 M^{-1} for the stability constant of $MgADP^{-}$ at PC equal to 20, 10 and 5 mM respectively. The reaction mixtures contained N-ethylmorpholine (pH 8.0), 0.1 M; creatine kinase, 0.576 µg. and EDTA (5×10^{-6} M) as well as the indicated amounts of Mg^{2+}, ADP^{3-} and PC. Total volume, 1.0 ml.; temp. 30°. Velocity is expressed as µmoles of creatine/µg. of creatine kinase/min.


PC = 20 mM
ADP 3−
0.066 mM
0.1 mM
0.2 mM
1/V
30
20
10
−4 −3 −2 −1 1 2 3 4 5
4x10⁻⁴ M / Mg2+


PC = 10 mM
ADP 3−
0.066 mM
0.1 mM
0.2 mM
0.4 mM
1/V
30
20
10
−5 −4 −3 −2 −1 1 2 3 4 5
4x10⁻⁴ M / Mg2+

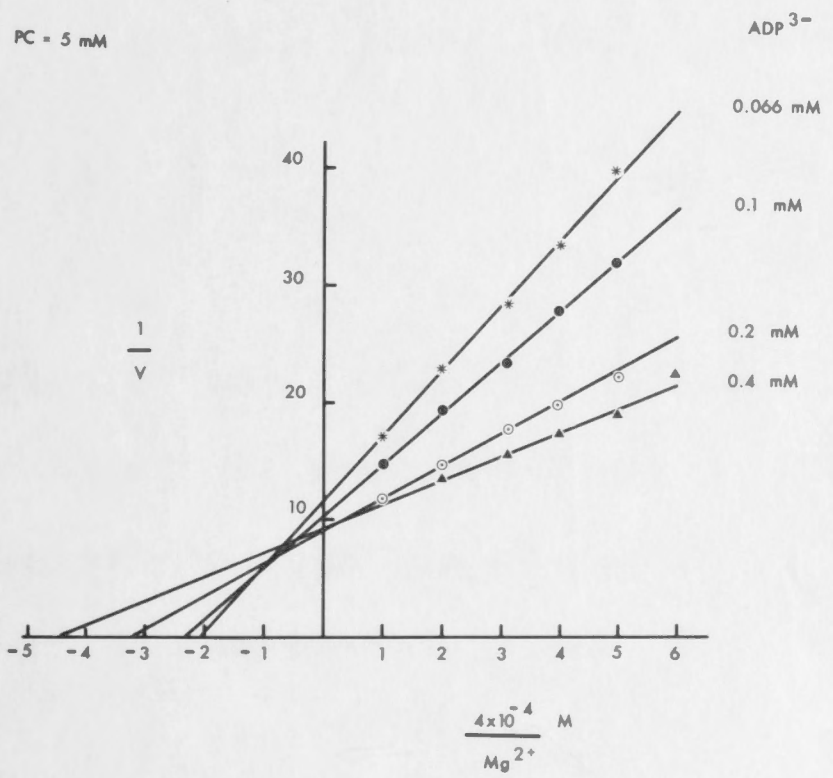

PC = 5 mM
ADP 3−
0.066 mM
0.1 mM
0.2 mM
0.4 mM
1/V
40
30
20
10
−5 −4 −3 −2 −1 1 2 3 4 5 6
4x10⁻⁴ M / Mg2+

TABLE 26

Variation of $K_{\sigma'}$ and $V_{\sigma'}$ with PC concentration

Values of $K_{\sigma'}$ and $V_{\sigma'}$ calculated from the plots in Fig.31 of $^1/v$ against $^1/Mg^{2+}$ at various fixed concentrations of ADP^{3-}. V expressed as μmoles creatine/μg. of creatine kinase/min.; K as mM.

ADP^{3-} (mM)	PC = 5 mM		PC = 10 mM		PC = 20 mM	
	$K_{\sigma'}$	$V_{\sigma'}$	$K_{\sigma'}$	$V_{\sigma'}$	$K_{\sigma'}$	$V_{\sigma'}$
0.4	0.083	0.108	0.082	0.137	-	-
0.2	0.124	0.115	0.136	0.152	0.119	0.163
0.1	0.171	0.099	0.176	0.137	0.198	0.152
0.066	0.187	0.086	0.230	0.139	0.272	0.150

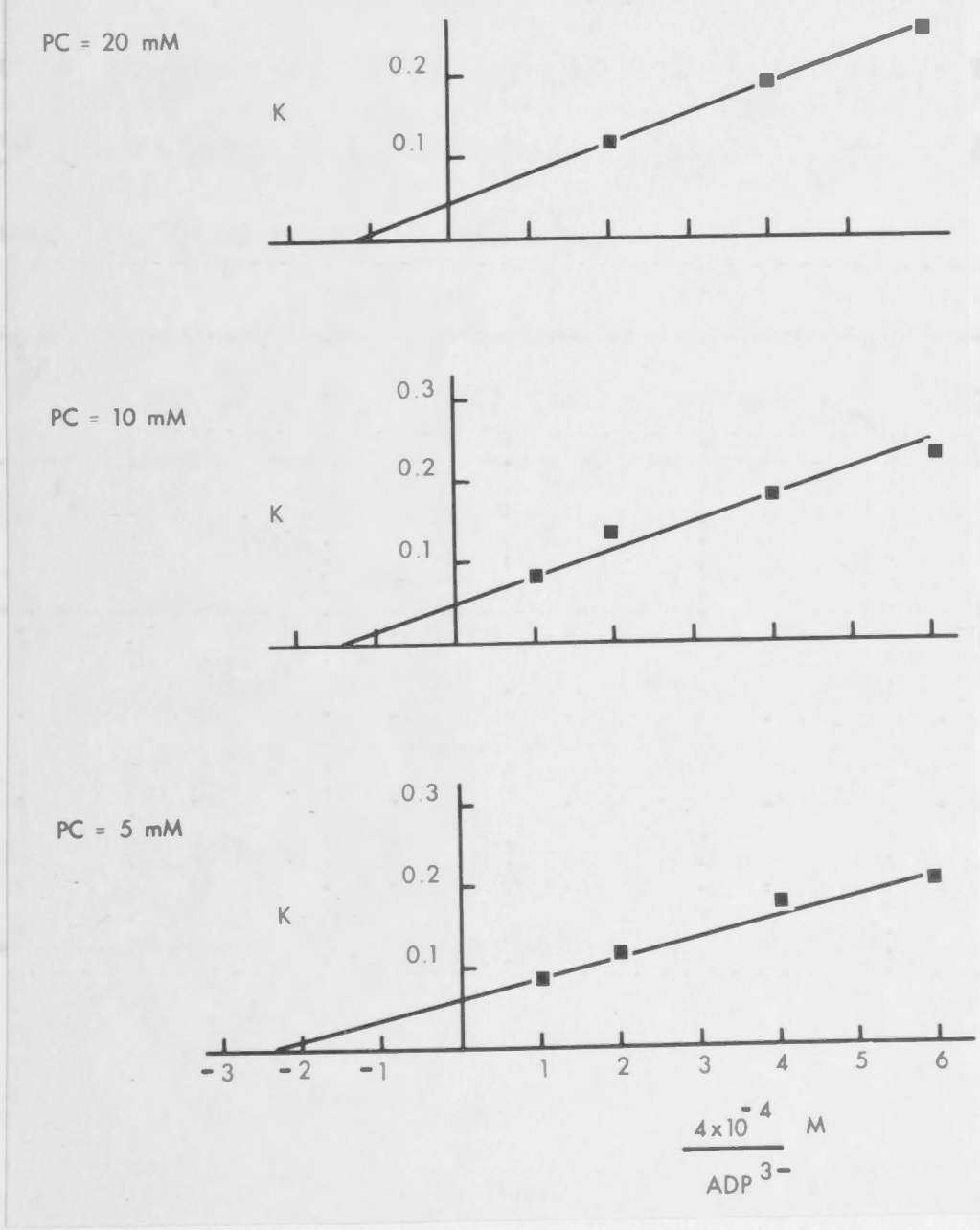


Fig. 32. Plot of the calculated values of $K_{\sigma'}$ against the reciprocal of the concentrations of ADP^{3-} (Eqn. 10). $K_{\sigma'}$ values obtained from the plots of $^{1}/v$ against $^{1}/Mg^{2+}$ at various fixed concentrations of ADP^{3-} (Fig. 31).

TABLE 27

Summary of the dissociation constants obtained if Mg^{2+} is assumed not to react with the enzyme

Constants calculated from plots of $K_{\sigma'}$ against $^{1}/ADP^{3-}$ (Fig. 32) and the relationship $K_1K_2 = K_5K_6$. All values expressed in terms of M x 10^4.

	PC = 5 mM	PC = 10 mM	PC = 20 mM
K_5	2.65	2.8	3.0
K_1	1.7	2.8	3.8
K_2	0.6	0.5	0.4
K_6	0.4	0.5	0.5

significant, as only four points at PC = 5 and 10 mM, and three points at PC = 20 mM were used in plotting K_σ' against $^1/_{\sigma'}$ and the experimental error was large.

At both 5 mM and 20 mM concentrations of PC there appeared to be a slight trend in the V_σ' values. Thus, in these cases it was possible to obtain values for all the constants, using plots of $^1/V_{\sigma'}$ against $^1/_{\sigma'}$ (Eqn. 7), and of $K_\sigma' \left(\frac{K_4}{\sigma'} + 1\right)$ against $^1/_{\sigma'}$ (Eqn. 8) (Table 28). Again these values, especially for K_3 and K_4, would be subject to large experimental error. Such calculations were not possible with the results obtained at PC = 10 mM, as the variation in the V_σ' values was random.

A plot of the reciprocal of the maximum velocity at each concentration of PC, against the reciprocal of the PC concentration, according to equation (4) is shown in Fig. 33. This plot gave a value of 4 mM for the K_m for PC, while the maximum velocity was found to be 0.20 μmoles creatine/μg. of creatine kinase/min. (16,200 moles/min./mole of enzyme) at pH 8.0, with the enzyme saturated with ADP^{3-}, Mg^{2+} and PC.

It was possible to plot the data shown in Fig. 31 as $^1/v$ against $^1/ADP^{3-}$, at different fixed concentrations of Mg^{2+}. In this case values were obtained for all the dissociation constants (Table 29a).

TABLE 28

Summary of the dissociation constants using individual values of $V_{\sigma'}$

Constants calculated from plots of $^1/V_{\sigma'}$ against $^1/_{\sigma'}$, and of $K_{\sigma'}$, $(\frac{K_4}{\sigma'} + 1)$ against $^1/_{\sigma'}$, using the values (Table 26) calculated from the plots of $^1/v$ against $^1/Mg^{2+}$ at various fixed values of ADP^{3-} (Fig. 31). All values expressed in terms of M x 10^4.

	PC = 5 mM	PC = 20 mM
K_5	2.65	3.0
K_1	2.5	3.8
K_2	0.6	0.4
K_3	10	>30
K_4	0.2	< 0.05
K_6	0.7	0.5

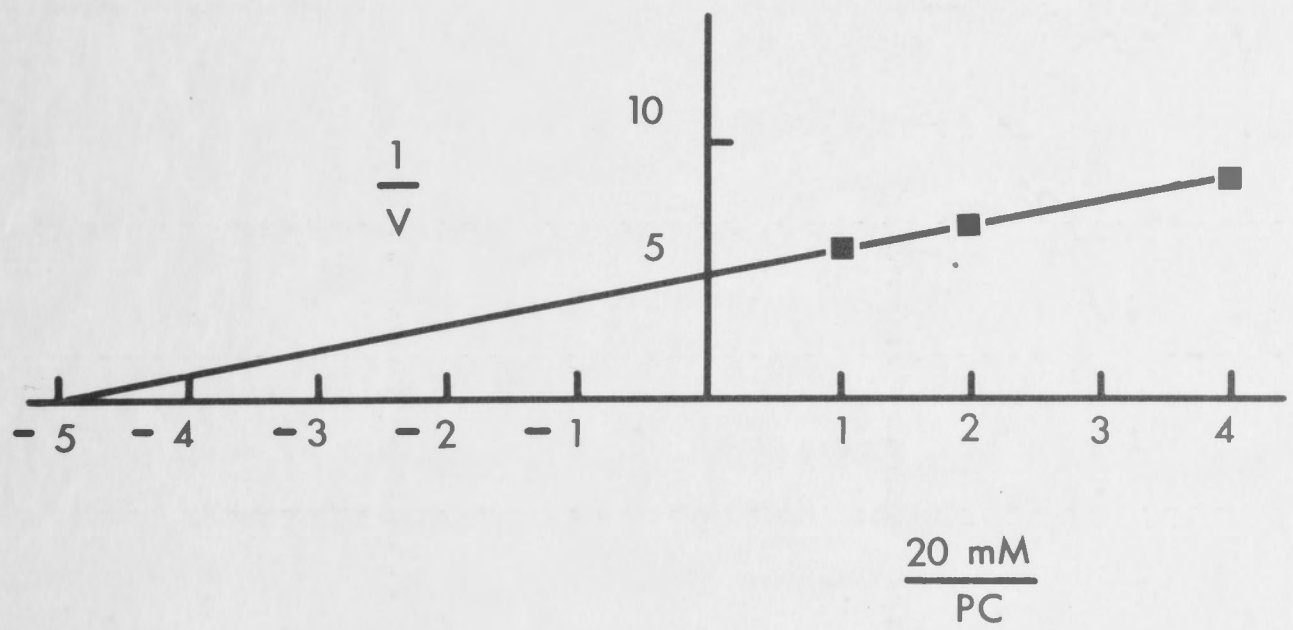


Fig. 33. Plot of the reciprocal of the maximum velocity at each PC concentration, from the data in Table 26, against the reciprocal of the PC concentration. (The average value of $V_{\sigma'}$ was used with PC = 10 mM. At PC = 5 and 20 mM, the $V_{\sigma'}$ values obtained by extrapolating to saturating concentrations of ADP^{3-} were used).

TABLE 29

Summary of the dissociation constants leading to the formation of an active EMS complex

(a) Values obtained from the data shown in Fig. 31, with $^1/v$ plotted against $^1/ADP^{3-}$ at different fixed concentrations of Mg^{2+}. All K values expressed in terms of M x 10^4.

	PC = 5 mM	PC = 10 mM	PC = 20 mM
K_1	1.0	1.5	2.0
K_2	0.8	0.8	0.8
K_3	1.8	7.8	5.7
K_4	0.45	0.2	0.3
K_6	0.3	0.4	0.5

(b) Values calculated from the plots of $^1/v$ against $^1/ADP^{3-}$ for various fixed values of Mg^{2+} (Fig. 34), using the V_μ, and K_μ, values recorded in Table 30. Estimates of the dissociation constants obtained from the same data, by plotting $^1/v$ against $^1/Mg^{2+}$ for various fixed values of ADP^{3-}, are also included. All K values expressed in terms of M x 10^4. PC = 10 mM.

	$^1/v$ against $^1/ADP^{3-}$	$^1/v$ against $^1/Mg^{2+}$
K_1	1.98	2.4
K_2	0.75	0.6
K_3	7.0	9.6
K_4	0.2	0.15
K_6	0.5	0.5

A further experiment was carried out, at PC = 10 mM, with Mg^{2+} held constant at various concentrations, this being the procedure originally adopted in Chapter IV. Plots of $^1/v$ against $^1/ADP^{3-}$ are shown in Fig. 34 and the calculated values of K_μ, and V_μ, are collected in Table 30. Plots of $^1/V_\mu$, against $^1/Mg^{2+}$ and of the function K_μ, $(\frac{K_2}{\mu'} + 1)$ against $^1/Mg^{2+}$ are shown in Fig. 35a and 35b respectively. Because it was evident from these plots that real values could be assigned to K_3 and K_4, the results shown in Table 30 have been obtained by the fine adjustment technique described by Wilkinson (1961) and the standard errors are included. The estimates of the dissociation constants obtained are shown in Table 29b. This same data was also treated by plotting $^1/v$ against $^1/Mg^{2+}$ at various fixed concentrations of ADP^{3-} and the dissociation constants obtained using this plot are included in Table 29b.

If $K_4 = 0$ and $K_3 = \infty$, then it would have been expected that the plot of K_μ, $(\frac{K_2}{\mu'} + 1)$ against $^1/\mu'$ (Fig. 35b) would have gone through the origin. This would appear to be unlikely though it is possible that the deviation of K_4 from zero is within the standard error. The evaluation of the standard error for this plot and of the probability that the line drawn could

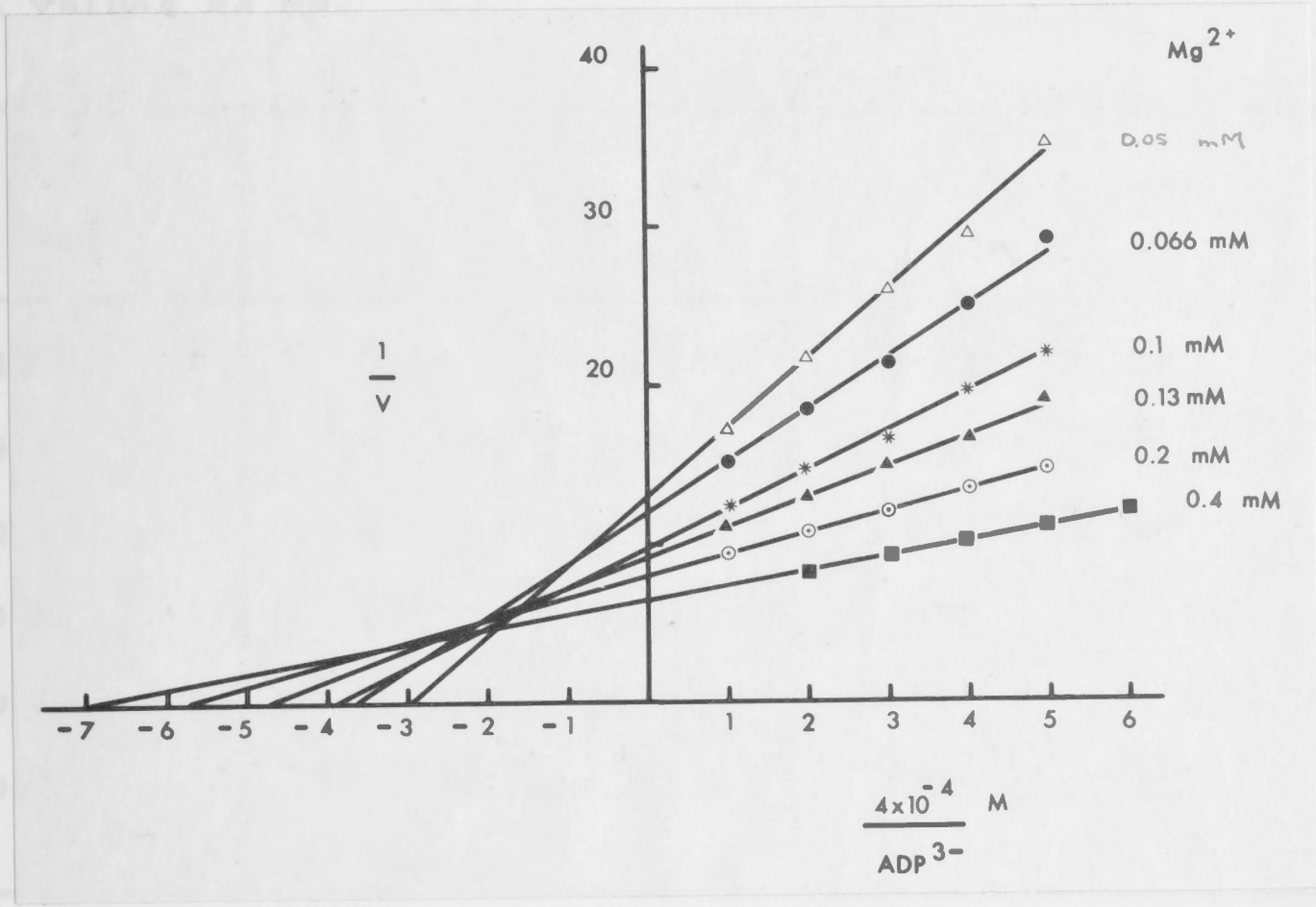


Fig. 34. Effect of the concentration of ADP^{3-} on the initial velocity of the reverse reaction, at various fixed concentrations of Mg^{2+}, for PC = 10 mM. Conditions identical to those for Fig. 31. Velocity is expressed as μmoles creatine/μg. creatine kinase/min.

TABLE 30

Summary of $V_{\mu'}$ and $K_{\mu'}$ values

Values calculated from the plots of $^1/v$ against $^1/ADP^{3-}$ at different fixed values of Mg^{2+} (Fig. 34). V values expressed as μmoles creatine/μg. creatine kinase/min.; K values as mM.

μ' (mM)	$V_{\mu'}$	$K_{\mu'}$
0.4	0.153 ± 0.009	0.0591 ± 0.0103
0.2	0.1263 ± 0.0151	0.0693 ± 0.0264
0.13	0.1083 ± 0.0353	0.0838 ± 0.0080
0.1	0.0967 ± 0.0045	0.0873 ± 0.0114
0.066	0.0839 ± 0.0045	0.1113 ± 0.0149
0.05	0.0773 ± 0.0028	0.1329 ± 0.0111

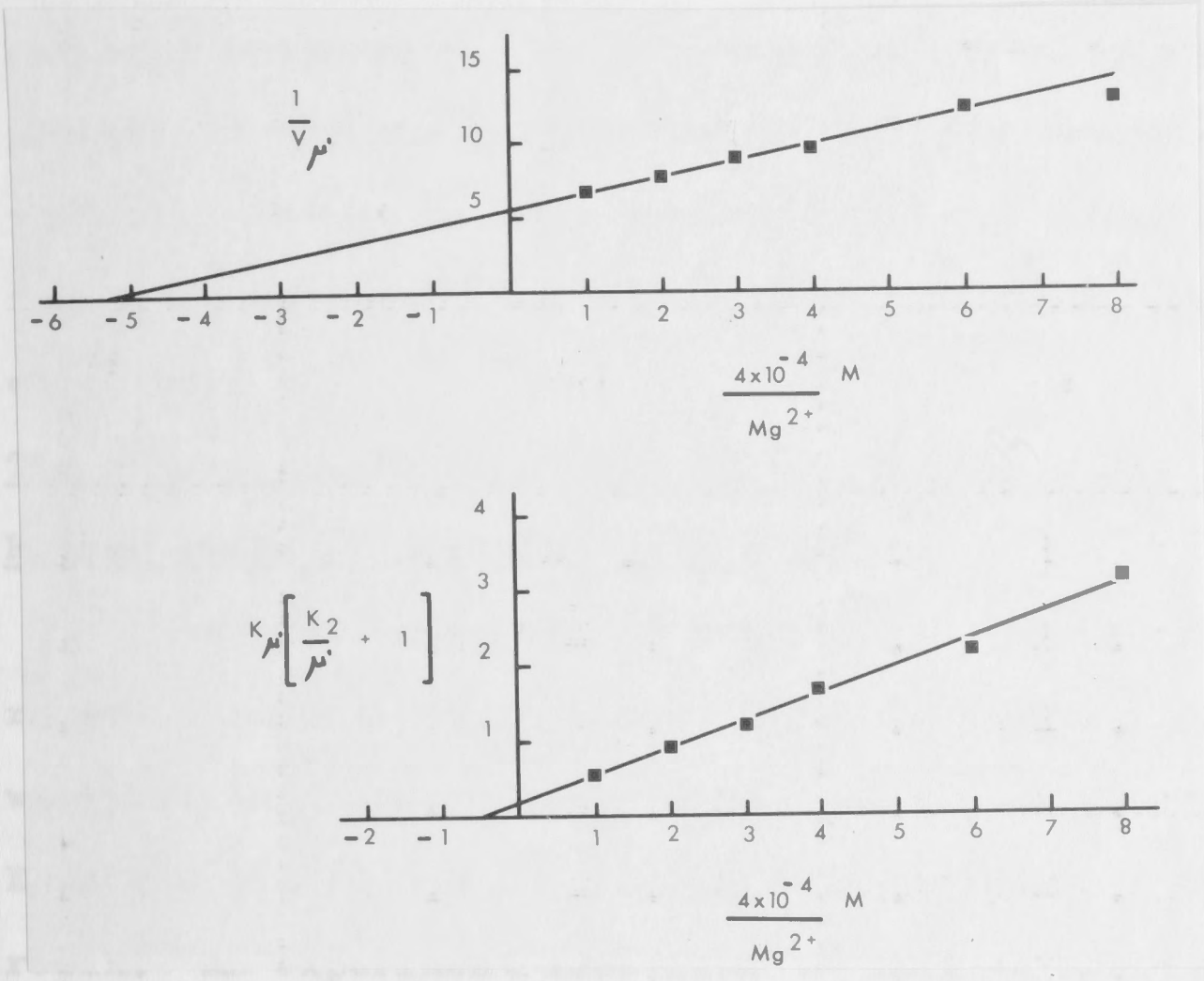


Fig. 35 (a). Plot of the reciprocals of the maximum velocities obtained in the presence of fixed amounts of Mg^{2+} and saturating amounts of ADP^{3-} (Fig. 34, Table 30) against the reciprocals of the Mg^{2+} concentrations. Maximum velocities expressed as μmoles creatine/μg. creatine kinase/min.

(b). Plot of the calculated values of $K_{\mu'}\left(\frac{K_2}{\mu'} + 1\right)$ against the reciprocals of the Mg^{2+} concentrations.

pass through the origin has not been attempted as this would have been very time consuming. Professor P. A. Moran of the Department of Statistics, Australian National University, has expressed the opinion that the probability of the plot shown in Fig. 35b passing through the origin would be very low, though it would be possible for K_4 to be lower, and hence K_3 higher, than the values obtained.

<u>The effect of omitting EDTA on the activation of creatine kinase by Mg^{2+} in relation to ADP^{3-}.</u>

The only experimental difference between the results reported in this chapter and those recorded in Chapter IV was that the former were carried out in the presence of EDTA and the PC and ADP had been pre-treated with Chelex resin. An experiment identical to that in Fig. 31, at PC = 10 mM, was carried out with EDTA omitted from the reaction mixtures.

Plots of $^1/v$ against $^1/Mg^{2+}$ are shown in Fig. 36. It is seen that the lines of best fit cut on the negative side of the y-axis. The scatter was much greater than in the presence of EDTA and the linearity was not as good. The results obtained at ADP^{3-} = 0.066 mM were not satisfactory and only the first two points were used in estimating $K_{\sigma'}$ and $V_{\sigma'}$ (dotted line in Fig. 36). Thus, it appeared that the Chelex treatment of PC and ADP was

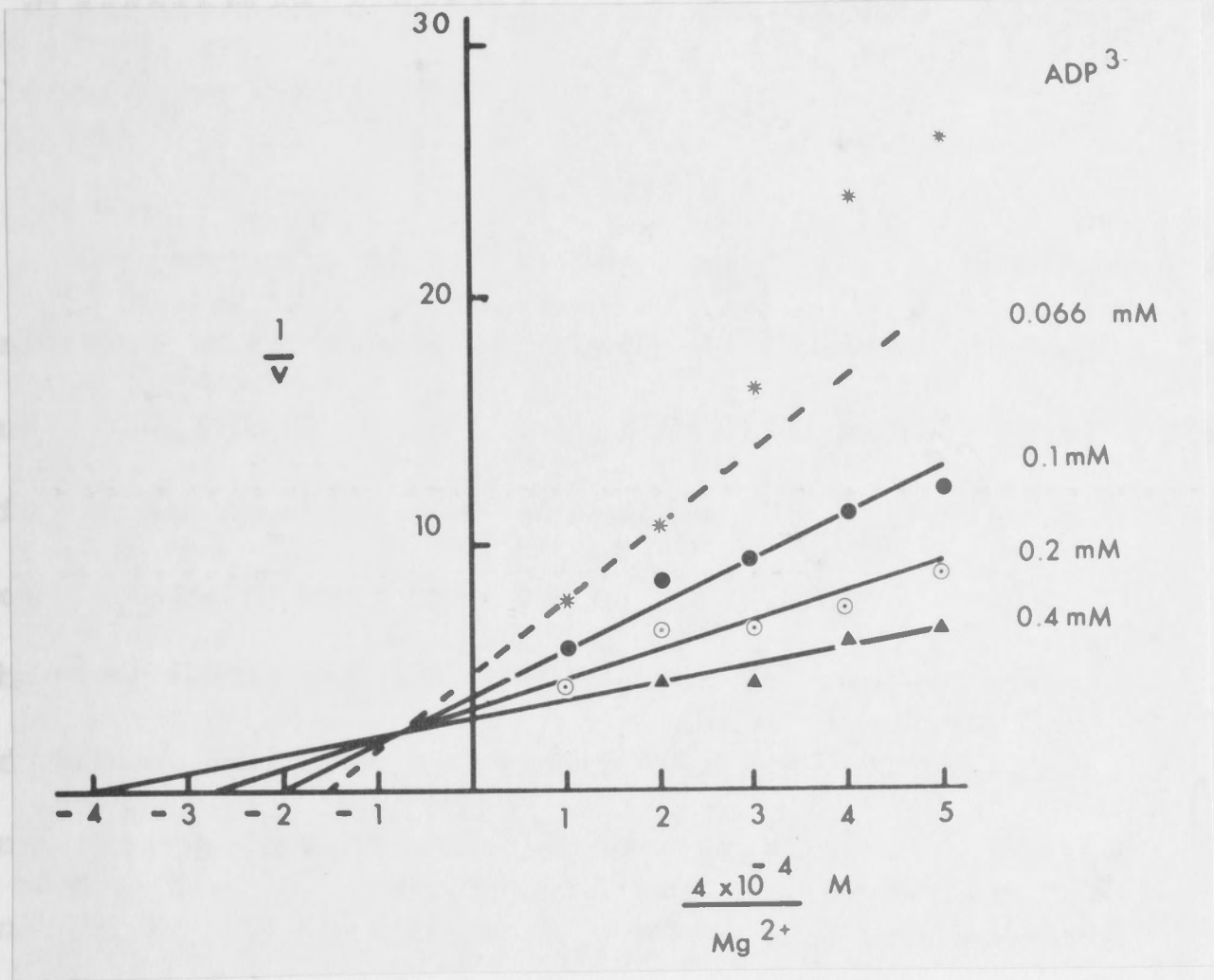


Fig. 36. Effect of the concentration of Mg^{2+} on the initial velocity of the reverse reaction, at various fixed concentrations of ADP^{3-}, for PC = 10 mM. Conditions the same as for Fig. 31 except that EDTA was not included. Velocities expressed as μmoles creatine/μg. creatine kinase/min.

insufficient to eliminate the time dependent deterioration of the enzyme.

Secondary and tertiary plots were made to determine the dissociation constants and the values obtained are collected in Table 31.

DISCUSSION

In general, the results reported in this chapter are consistent with those obtained in Chapter IV using the value of 4,150 M^{-1} for the stability constant of $MgADP^-$ and taking into account the trend in the $K_{\mu'}$ values. It is almost certain that the value of 1,000 M^{-1} for the stability constant of $MgADP^-$, under the experimental conditions, is incorrect and thus the results obtained using this constant should be discounted. Further, the trend in $K_{\mu'}$ with μ' (or $K_{\sigma'}$ with σ') has been confirmed and it has been seen that it is essential to use the individual K values for the tertiary plots (e.g., Eqn. 8).

The values determined for K_3, the constant for the interaction between the enzyme and Mg^{2+}, were usually lower than the values found from the inhibition experiments. The latter had given a K_i for Mg^{2+} as being between 10 and 20 mM. This is not significantly different from the value of infinity, which could be obtained from the plots of $^1/v$ against $^1/Mg^{2+}$ in Fig. 31, but is considerably higher than the other values reported in this chapter.

Particular attention is brought to the finding

TABLE 31

Summary of the dissociation constants

obtained in the absence of EDTA

Constants calculated from the plots of $^1/v$ against $^1/Mg^{2+}$, in the absence of EDTA (Fig. 36). All K values expressed in terms of M x 10^4. PC = 10 mM.

K_1	4.0
K_2	0.6
K_3	7.0
K_4	0.36
K_6	0.9

that a particular set of data could give slightly different values of the dissociation constants according to how the plots were carried out. Thus, the assumption that $K_3 = \infty$ (and hence $K_4 = 0$) appeared to be valid for the plots of $^1/v$ against $^1/Mg^{2+}$ (Fig. 31). Certainly for the data obtained with PC = 10 mM, effectively the same value of the maximum velocity was obtained at each fixed concentration of ADP^{3-}. At the other concentrations (5 and 20 mM) of PC used, there appeared to be a slight trend in the maximum velocities, though this trend would be barely outside the standard error. When the same data was plotted as $^1/v$ against $^1/ADP^{3-}$, definite values were obtained for both K_3 and K_4 (Table 29). This is also demonstrated in the direct plot of $^1/v$ against $^1/ADP^{3-}$ (Fig. 34). However, it is clear from the plot of $K_{\mu'}$ ($\frac{K_2}{\mu'} + 1$) against $^1/\mu'$ (Fig. 35b) that a relatively small variation in one of the experimental parameters would be sufficient to considerably reduce K_4 (and hence increase K_3), possibly to such an extent as to make it indistinguishable from zero and K_3 indistinguishable from infinity. An estimate of the overall standard error for the value of K_4 was considered too tedious to be undertaken in the time available. As noted above, and in Chapter IV, the values obtained for the dissociation constants are sensitive to the magnitude of the stability constant used

for $MgADP^-$ and it is possible that a relatively small error in this quantity could be responsible for the experimental results indicating real values of K_3 or K_4, or vice versa. For example, a discrepancy in the value of K_5 could either be responsible for such a trend as is shown in Fig. 31a and Fig. 31c, or for eliminating a possible trend to give the results observed in Fig. 31b.

If the values for K_3 and K_4 are real, then the results shown in Table 29 indicate that the presence of Mg^{2+} on the enzyme would appreciably effect the binding of ADP^{3-} ($K_1 > K_4$), while the presence of ADP^{3-} on the enzyme would have a similar effect on the binding of Mg^{2+} ($K_3 > K_2$).

The values obtained for K_1 and K_6 were in reasonable agreement with the equilibrium values obtained by Kuby, Mahowald and Noltmann (1962). Thus, for the interaction between ADP^{3-} and the enzyme, the values of K_1 reported in this chapter ranged from $1.7 - 4 \times 10^{-4}$ M, while Kuby *et al.* (1962) obtained an equilibrium value of 1×10^{-4} M. For $MgADP^-$, the kinetically determined value, K_6, lay between $0.4 - 0.7 \times 10^{-4}$ M, Kuby *et al.* (1962) reporting a value of $0.6 - 0.7 \times 10^{-4}$ M.

Finally, it is worth noting that the use of EDTA increased the experimental precision but did not qualitatively alter the interpretation of the experimental results.

SUMMARY

A reassessment of the kinetics of the reverse reaction catalysed by creatine kinase, using the values for the stability constant of $MgADP^-$ as reported in Chapter II and including low concentrations of EDTA, has been carried out. The results, in general, were consistent with a weak interaction between the metal ion and the enzyme, though in some experiments this interaction could be interpreted as being negligible.

CHAPTER VII

THE KINETICS OF THE FORWARD REACTION CATALYSED BY CREATINE KINASE

CHAPTER VII

THE KINETICS OF THE FORWARD REACTION CATALYSED BY CREATINE KINASE

INTRODUCTION

The results reported in this chapter must be regarded as being preliminary. While it was hoped to carry out an extensive study of the reaction in the forward direction, this has not been possible within the time available. Nevertheless, it is of interest to compare the results obtained so far with those previously reported from studies of the reverse reaction.

If, as the results in Chapter IV indicated, the formation of an active EMS complex, in the reverse reaction, could occur by the three possible pathways postulated, then it might be expected that a study under similar conditions of the reaction in the direction

ATP + creatine $\longrightarrow$ ADP + phosphorylcreatine

would yield similar results. This being so, it would be expected that the value obtained for the dissociation constant of an enzyme-metal complex would be the same as that determined from the reverse reaction studies.

Two sets of results, both studied at two pH values, carried out under slightly different experimental conditions, are described. In general, these experiments

were consistent with the formation of EMS by all three pathways, though one experiment could be interpreted as indicating that Mg^{2+} did not react with the enzyme.

THEORY

The same theoretical treatment as was elaborated in Chapters IV and VI has been applied. The same symbols are also used, except that in this chapter the symbol, σ, refers to the concentration of free nucleotide, ATP^{4-}.

MATERIALS

Creatine hydrate (British Drug Houses Laboratory Reagent) was twice recrystallised from water. The product was washed with ethanol and ether, dried in air and analysed as creatine.H_2O. (Found: N, 28.3; H_2O, 12.4: theory for creatine.H_2O: N, 28.2; H_2O, 12.1%).

Other materials were as described in Chapters II and III.

METHODS

ATP was passed through a Chelex resin to remove trace metals and standardised immediately before use.

Phosphorylcreatine was estimated as inorganic phosphate by a slight modification of the method described by Kuby *et al.* (1954a). To 1.0 ml. of the reaction mixture was added 0.9 ml. of acid molybdate solution (0.5 ml. of 5% (w/v) ammonium molybdate and

0.4 ml. of 60% (v/v) perchloric acid) and 0.3 ml. of 1% (w/v) ascorbic acid solution. The volume was made to 3.0 ml. and the optical density of the resulting blue solution determined at 750 mμ on a Shimadzu (QR-50) spectrophotometer after exactly 30 min. Under these conditions, the PC was totally hydrolysed and 0.1 μmole of PC gave an optical density of 0.150 when measured in a 1 cm. cell. Alternatively, for reactions carried out in 2.0 ml. of solution, the reaction was stopped by 0.7 ml. of acid-molybdate solution to give the same final concentration of ammonium molybdate and perchloric acid as above, 0.3 ml. of 1% ascorbic acid added and the solutions read at 750 mμ after 30 min.

Enzymic activity was determined at 30° in 3 ml. graduated tubes and the reaction mixture for the early experiments contained triethanolamine buffer (0.1 M), creatine (0.01 M) and various amounts of ATP and $MgCl_2$. In later experiments, the volume of the reaction mixture was increased to 2.0 ml., the creatine concentration to 0.02 M and, in addition, the solutions contained EDTA (5×10^{-6} M). After equilibration for 3 min., the reaction was started by the addition of 0.02 ml. of enzyme solution, equivalent to 2 - 3 μg. of protein. At the end of the incubation period ($\frac{1}{2}$ to $1\frac{1}{2}$ min.) the reaction was stopped by the addition of acid-molybdate

solution and the PC estimated as described above. A blank containing all constituents except enzyme was run with each estimation in order to allow for acid hydrolysis of ATP.

Experimental points are shown in all graphs unless otherwise indicated, but the lines have been drawn using V and K values calculated by Wilkinson's (1961) statistical method. In some cases the data have been subjected to the fine adjustment described by Wilkinson (1961) and the standard errors have been included.

RESULTS

In the studies reported in the previous chapters, the pH and ionic strength of the reaction mixtures were maintained by N-ethylmorpholine, which does not complex with divalent metal ions. As this buffer interfered with the estimation of phosphate, triethanolamine was substituted for the experiments reported below. The ability of this buffer to complex with Mg^{2+} was considered to be negligible (see Chapter II). The stability constant of $MgATP^{2-}$, as reported in Chapter II, was 70,000 M^{-1} (K_5 = 0.014mM) under the experimental conditions and this value was used to determine the concentrations of total Mg and total ATP required to maintain Mg^{2+} and ATP^{4-} at pre-determined levels.

Two sets of experiments, carried out under slightly

different conditions, are reported. Those experiments reported in Section I were carried out at pH 8.0 and pH 8.55 with the creatine concentration at 0.01 M. For the experiments described in Section II, the creatine concentration was increased to 0.02 M and EDTA was included in the reaction mixtures. The effect of including EDTA was to slightly increase both the activity of the enzyme and the linearity of the reaction, though its effect was much less profound than had been observed for the reverse reaction, presumably because ATP was a better chelator of trace metals than ADP. Its presence also served as a safeguard against the inadvertent introduction of contaminant metal inhibitors.

SECTION I.

The activation of creatine kinase by Mg^{2+} in relation to ATP^{4-} at pH 8.0.

The effect of the concentration of ATP^{4-} (σ) on the velocity of the reaction at pH 8.0 and at various fixed concentrations of Mg^{2+} (μ') is shown (Fig. 37) as a double reciprocal plot. The calculated values of $K_{\mu'}$ and $V_{\mu'}$ are collected in Table 32. The concentration of Mg^{2+} appears to have only a small and perhaps negligible effect on the magnitude of $K_{\mu'}$ over the range studied.

A straight line was obtained when $1/\mu'$ was plotted

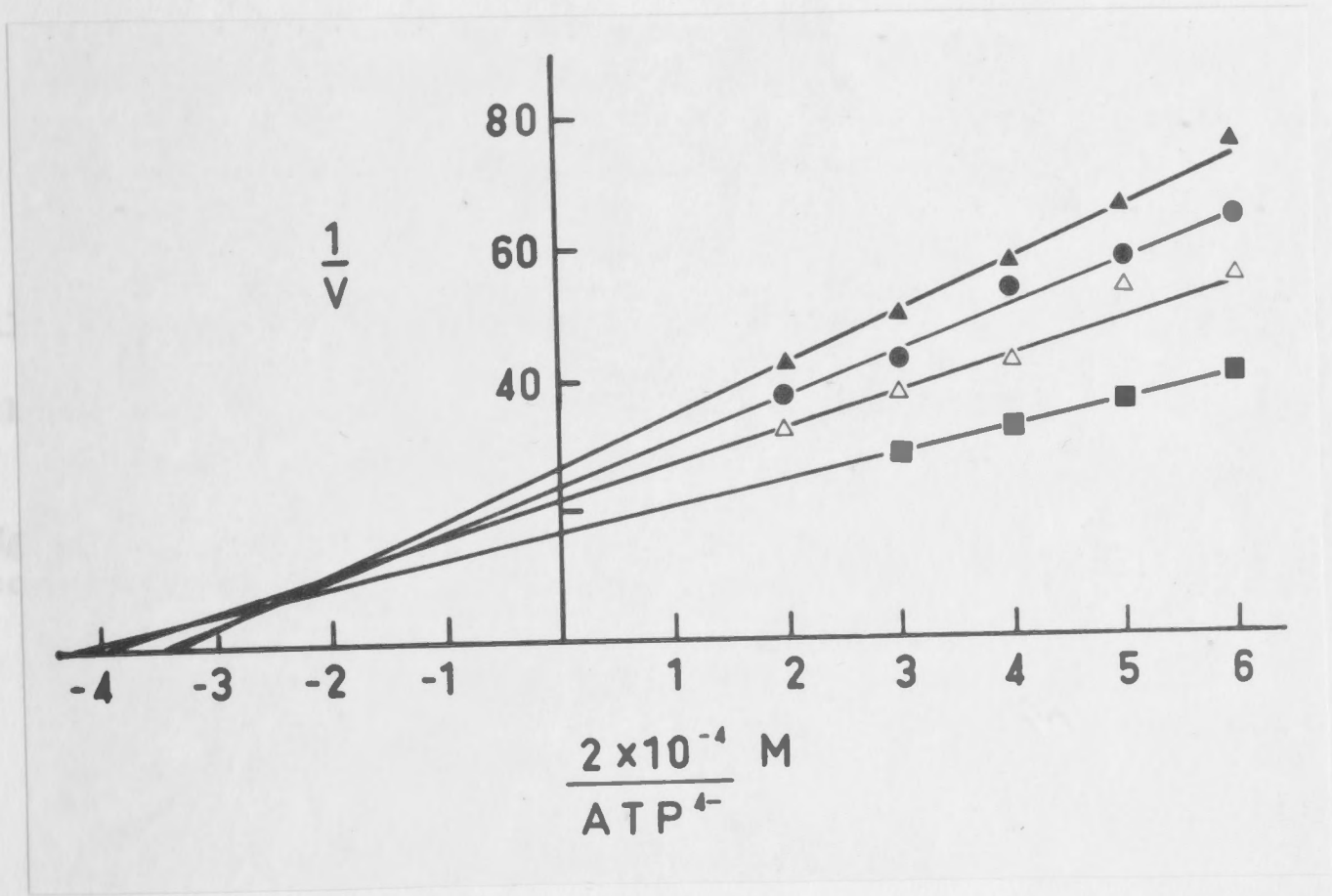


Fig. 37. Effect of the concentration of ATP^{4-} on the initial velocity of the reaction at various fixed values of Mg^{2+}. The concentrations of ATP^{4-} and Mg^{2+} were adjusted by varying the total concentrations of ATP and Mg^{2+} using a value of 70,000 M^{-1} for the stability constant of $MgATP^{2-}$. The reaction mixtures contained triethanolamine (pH 8.0), 0.1 M; creatine, 10^{-2} M, and enzyme, 3 µg., as well as the indicated amounts of Mg^{2+} and ATP^{4-}. Total volume, 1.0 ml.; temperature 30°. Velocity is expressed as µmoles of phosphorylcreatine formed/µg. of enzyme/min. ■—■, Mg^{2+}, 2×10^{-4} M; △—△, Mg^{2+}, 1×10^{-4} M; ●—●, Mg^{2+}, 0.66×10^{-4} M; ▲—▲, Mg^{2+}, 0.5×10^{-4} M.

TABLE 32

Values calculated from the plots of $^1/v$ against $^1/ATP^{4-}$ at various fixed levels of Mg^{2+} (μ')

(Fig. 37)

All K values are expressed in terms of M x 10^4, while those for $V_{\mu'}$ are given in arbitrary units.

Mg^{2+} concentration (μ')	$K_5 = 0.14$			
	$K_{\mu'}$		$V_{\mu'}$	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
2.0	0.47±0.03	0.43±0.04	2.70±0.08	2.28±0.11
1.0	0.50±0.09	0.39±0.22	2.03±0.14	1.78±0.40
0.66	0.57±0.10	0.61±0.06	1.88±0.16	1.81±0.09
0.50	0.59±0.07	0.46±0.06	1.73±0.10	1.33±0.08
Average value of $K_{\mu'}$	0.53	0.52		

against $^{1}/V_{\mu'}$ (Fig. 38a) and the value for K_2 was calculated to be 0.54 ± 0.08 x 10^{-4} M. The duplicate experiment in Table 32 gave K_2 at 0.48 ± 0.10 x 10^{-4} M. Because of the possibility that the effect of the Mg^{2+} concentration on the $K_{\mu'}$ values might have been real, values for K_4 and K_4/K_1K_2 were determined from plots of $^{1}/\mu'$ against $K_{\mu'}$ $(\frac{K_2}{\mu'} + 1)$, for which both the average and individual values of $K_{\mu'}$ were used (Fig. 38b). The dissociation constants obtained are collected in Table 33.

Activation of creatine kinase by Mg^{2+} in relation to ATP^{4-} at pH 8.55.

Identical kinetic experiments were carried out at pH 8.55. Because the velocity of the reaction is much faster and the equilibrium more favourable at this pH than at pH 8.0, it was possible to determine the initial rates with greater accuracy.

Plots of $^{1}/v$ against $^{1}/ATP^{4-}$ for different values of free Mg^{2+} (μ') are shown in Fig. 39 and the calculated values of $K_{\mu'}$ and $V_{\mu'}$ in Table 34, together with the results of a duplicate experiment. In contrast to the results obtained at pH 8.0, the $K_{\mu'}$ values tend to increase with decreasing free Mg^{2+} concentration. The values obtained for the dissociation constants were calculated using the individual values of $K_{\mu'}$ and are included in Table 33.

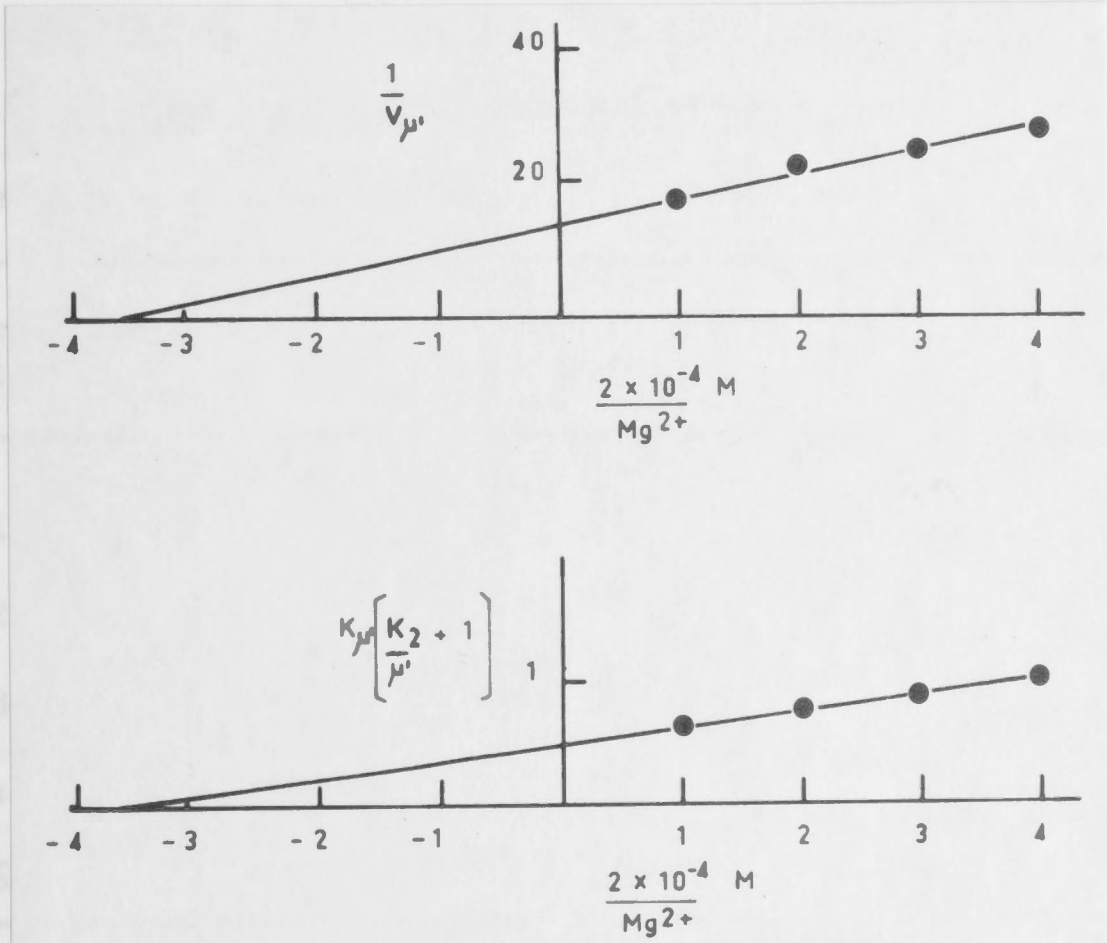


Fig. 38(a). Plot of the reciprocals of the maximal velocities obtained in the presence of fixed amounts of Mg^{2+} and saturating amounts of ATP^{4-}, against the reciprocals of the concentrations of Mg^{2+}. Maximum velocity values were obtained from the plot shown in Fig. 37 and are expressed as μmoles of phosphorylcreatine formed/μg. of enzyme/min.

(b). Plot of the calculated values for $K_{\mu'}$ $(K_2/\mu' + 1)$ against the reciprocals of the concentrations of Mg^{2+}.

TABLE 33

Summary, from the experiments described in Section I, of the dissociation constants for the various reactions leading to the formation of the active enzyme-metal-substrate complex

All values are expressed in terms of M x 10^4.

Dissociation constants	pH 8.0				pH 8.55
	(a)	(b)	(c)	(d)	
K_1	0.53	0.52	0.63	0.71	1.51
K_2	0.54	0.48	0.54	0.51	0.31
K_3	0.54	0.48	0.79	1.00	1.22
K_4	0.53	0.52	0.43	0.36	0.38
K_6	2.04	1.78	2.43	2.57	3.28

The values shown in columns (a) - (d) were obtained from the data given in Table 32. Columns (a) and (b) represent the dissociation constants calculated from the average value of $K_{\mu'}$ for each series, together with the values of 0.54×10^{-4} M (a) and 0.48×10^{-4} M (b) for K_2. Column (c) represents the values obtained from Exp. 1, using the individual values of $K_{\mu'}$ at each concentration of Mg^{2+} (μ') and a value of 0.54×10^{-4} M for K_2. Column (d) shows the calculated results using the average of each of the two $K_{\mu'}$ values obtained at each concentration of Mg^{2+} and an average value of 0.51×10^{-4} M for K_2.

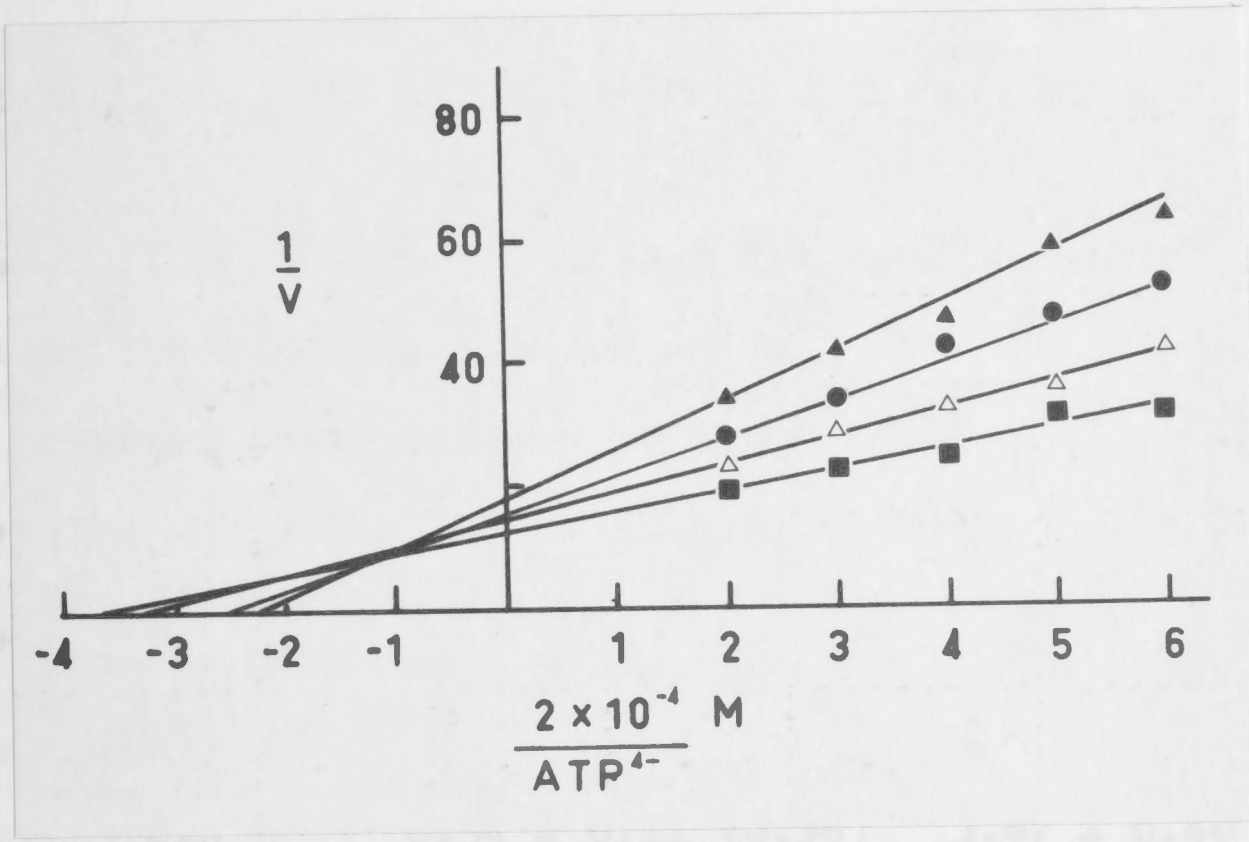


Fig. 39. Effect of the concentration of ATP^{4-} on the initial velocity of the reaction at various fixed values of Mg^{2+} at pH 8.55. The concentrations of ATP^{4-} and Mg^{2+} were adjusted by varying the total concentrations of ATP and Mg^{2+} using a value of 70,000 M^{-1} for the stability constant of $MgATP^{2-}$. The reaction mixtures contained triethanolamine (pH 8.0), 0.1 M; creatine, 10^{-2} M, and enzyme, 3 μg., as well as the indicated amounts of Mg^{2+} and ATP^{4-}. Total volume, 1.0 ml.; temperature 30°. Velocity is expressed as μmoles of phosphorylcreatine formed/μg. of enzyme/min. ■——■, Mg^{2+}, 2×10^{-4} M; △——△, Mg^{2+}, 1×10^{-4} M; ●——●, Mg^{2+}, 0.66×10^{-4} M; ▲——▲, Mg^{2+}, 0.5×10^{-4} M.

TABLE 34

Summary of the values obtained for $K_{\mu'}$ and $V_{\mu'}$ at pH 8.55

Values calculated from plots of $^1/v$ against $^1/ATP^{4-}$ at various fixed concentrations of Mg^{2+} (μ') (Fig. 39). All K values are expressed in terms of M x 10^4, while those for $V_{\mu'}$ are given in arbitrary units. K_5 = 0.14.

Mg^{2+} concentration (μ')	$K_{\mu'}$	$V_{\mu'}$
2.0	0.54 ± 0.11 (0.36)	3.87 ± 0.40 (3.15)
1.0	0.60 ± 0.09 (0.70)	3.11 ± 0.25 (3.28)
0.66	0.75 ± 0.07 (0.79)	3.06 ± 0.17 (2.98)
0.5	0.87 ± 0.16 (0.84)	2.74 ± 0.30 (2.61)

Figures in parentheses represent results from a duplicate experiment.

SECTION II.

The activation of creatine kinase by ATP^{4-} in relation to Mg^{2+} at pH 8.0.

For the experiments carried out in the presence of EDTA $^{1}/v$ was plotted against $^{1}/Mg^{2+}$ for various fixed concentrations of ATP^{4-} (Fig. 40). The calculated values of $K_{\sigma'}$ and $V_{\sigma'}$ are collected in Table 35. These show that there is a slight trend in the $K_{\sigma'}$ values and that the maximum velocity is not independent of the concentration of ATP^{4-}, as would have been expected if there was no interaction between Mg^{2+} and the enzyme (cf., Chapter VI).

An estimate of K_4 was obtained from a plot of $^{1}/V_{\sigma'}$ against $^{1}/\sigma'$ (Fig. 41a) and of K_1 and K_2 from a plot of $K_{\sigma'}$ $(\frac{K_4}{\sigma'} + 1)$ against $^{1}/\sigma'$ (Fig. 41b). Thus, values were obtained for all the dissociation constants (Table 36). The maximum velocity was also estimated from Fig. 41a as being 0.298 μmoles PC/min./μg. creatine kinase (24,000 moles PC/min./mole of enzyme), at pH 8.0 when the enzyme is saturated with ATP^{4-} and Mg^{2+} and the creatine concentration is 20 mM.

The activation of creatine kinase by ATP^{4-} in relation to Mg^{2+} at pH 8.4.

Plots of $^{1}/v$ against $^{1}/Mg^{2+}$, for various fixed concentrations of ATP^{4-}, at pH 8.4 are shown in Fig. 42

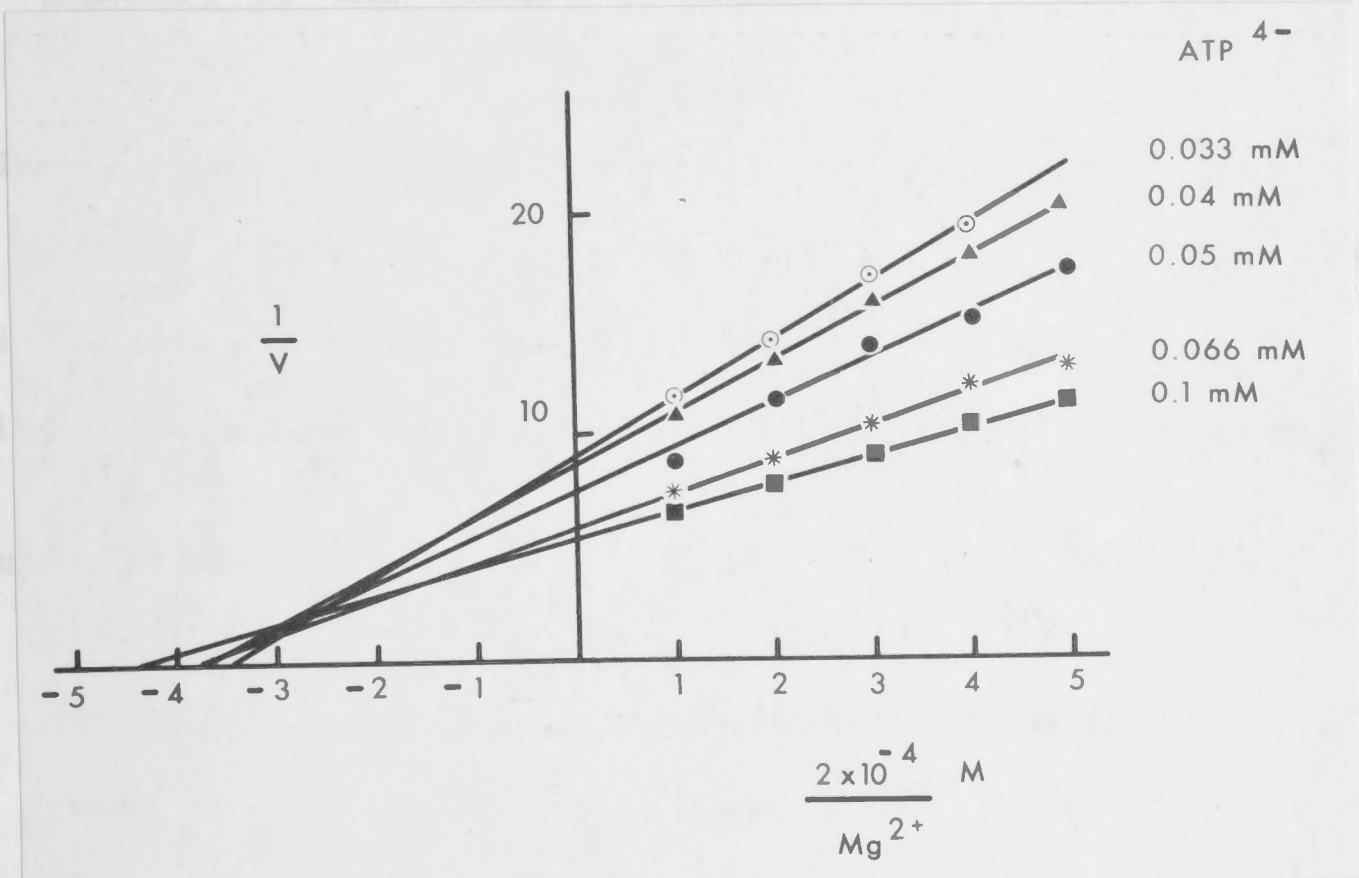


Fig. 40. Effect of the concentration of Mg^{2+} on the initial velocity of the reaction at various fixed values of ATP^{4-}. The concentrations of ATP^{4-} and Mg^{2+} were adjusted by varying the total concentrations of ATP and Mg^{2+} using a value of 70,000 M^{-1} for the stability constant of $MgATP^{2-}$. The reaction mixtures contained triethanolamine (pH 8.0), 0.1 M; creatine 2×10^{-2} M; EDTA (5×10^{-6} M) and creatine kinase (2.3 µg) as well as the indicated amounts of Mg^{2+} and ATP^{4-}. Total volume, 2.0 ml; temperature 30°. Velocity is expressed as µmoles PC/min./µg creatine kinase.

TABLE 35

Summary of the values obtained for $K_{\sigma'}$ and $V_{\sigma'}$ at pH 8.0

Values calculated from the plots of $^1/v$ against $^1/Mg^{2+}$ at various fixed concentrations of ATP^{4-} (σ') (Fig. 40). All K values are expressed in terms of M x 10^4 and V values as μmoles PC released/min./μg. creatine kinase.

ATP^{4-} concentration (σ')	$K_{\sigma'}$	$V_{\sigma'}$
1.0	0.46	0.181
0.66	0.53	0.171
0.5	0.53	0.131
0.4	0.53	0.112
0.33	0.57	0.109

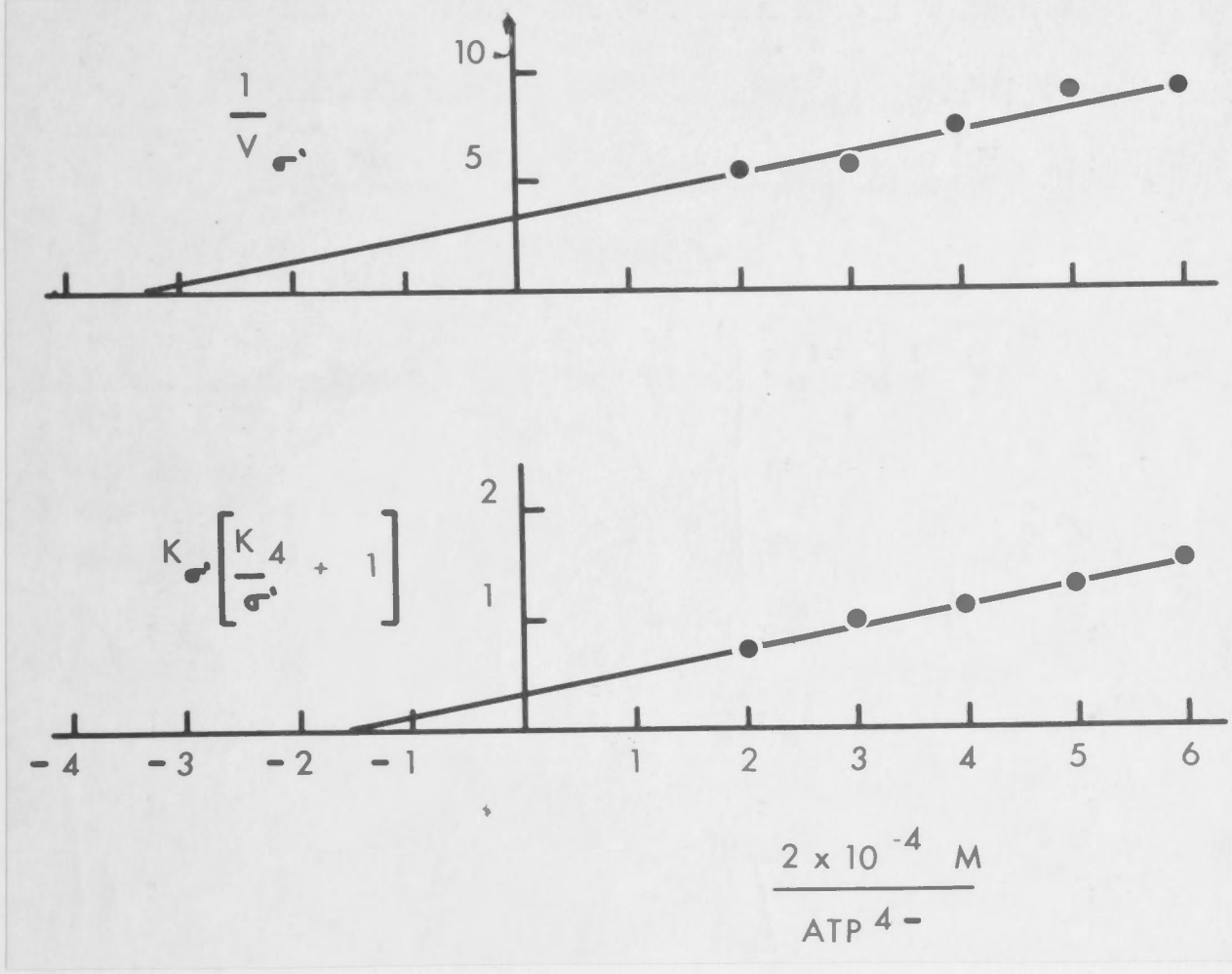


Fig. 41. (a) Plot of the reciprocals of the maximal velocities obtained in the presence of fixed amounts of ATP^{4-} and saturating amounts of Mg^{2+}, against the reciprocals of the concentrations of ATP^{4-}. Maximum velocities were obtained from the plots shown in Fig. 40 and are expressed as μmoles of phosphorylcreatine formed/μg. of enzyme/min.

(b) Plot of the calculated values for $K_{\sigma'}(K_4/\sigma' + 1)$ against the reciprocals of the concentrations of ATP^{4-}.

TABLE 36

Summary from the experiments described in Section II of the dissociation constants for the various reactions leading to the formation of the active enzyme-metal-substrate complex

All values expressed in terms of M x 10^4; K_5 = 0.14 x 10^4 M.

Dissociation constants	pH 8.0	pH 8.4	
K_1	1.22	0.55	(0.7)*
K_2	0.33	0.26	(0.28)
K_3	0.68		(5)
K_4	0.59		(0.04)
K_6	2.88	1.02	(1.4)

*Values in parenthesis obtained from plotting the results at pH 8.4 as $^1/v$ against $^1/ATP^{4-}$.

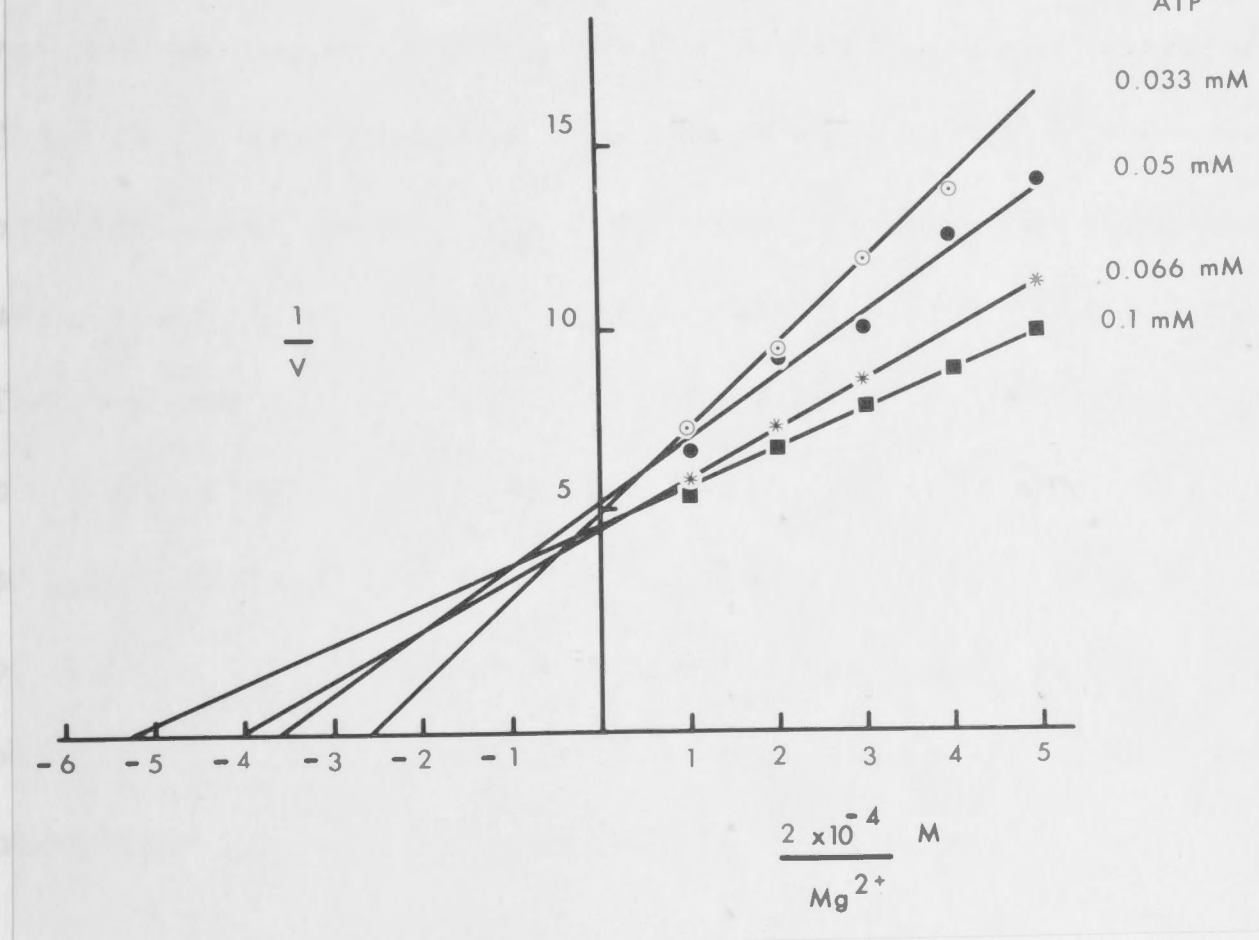


Fig. 42. Effect of the concentration of Mg^{2+} on the initial velocity of the reaction at various fixed values of ATP^{4-}. The concentrations of ATP^{4-} and Mg^{2+} were adjusted by varying the total concentrations of ATP and Mg^{2+} using a value of 70,000 M^{-1} for the stability constant of $MgATP^{2-}$. The reaction mixtures contained triethanolamine (pH 8.4), 0.1 M; creatine 2×10^{-2} M; EDTA (5×10^{-6} M) and creatine kinase (2.3 µg) as well as the indicated amounts of Mg^{2+} and ATP^{4-}. Total volume, 2.0 ml; temperature 30°. Velocity is expressed as µmoles PC/min./µg creatine kinase.

and the calculated values of $K_{\sigma'}$ and $V_{\sigma'}$ in Table 37. In this case, there is a definite trend in the $K_{\sigma'}$ values and the values of $V_{\sigma'}$ appear to be independent of σ', the variation being random and within the experimental error. This fits the form of the general equation for the condition $K_3 = \infty$, $K_4 = 0$, so that Eqn. 9, Chapter VI, may be applied and $K_{\sigma'}$ plotted against $^1/\sigma'$ (Fig. 43). The values of the dissociation constants obtained from this plot are included in Table 36. It was also possible to plot this data as $^1/v$ against $^1/ATP^{4-}$ for fixed values of Mg^{2+}. In this case a small but real value of K_4 was obtained. The dissociation constants obtained from this plot are shown in parentheses in Table 36.

DISCUSSION

The kinetic studies of the forward reaction catalysed by creatine kinase have been severely hampered by the insensitivity of the assay procedure. Further, the reaction is only linear for short time periods (1 to $1\frac{1}{2}$ min.). The linearity of the reaction and the activity of the enzyme were both slightly increased by including EDTA in the reaction mixtures but the effect was much less profound than had been observed with the reverse reaction.

The results obtained at pH 8.0 in Section I (Table 33) are in general agreement with the conclusions

TABLE 37

Summary of the values obtained for $K_{\sigma'}$ and $V_{\sigma'}$ at pH 8.4

Values calculated from the plots of $^1/v$ against $^1/Mg^{2+}$ at various fixed levels of ATP^{4-} (σ') (Fig. 42). All K values are expressed in terms of M x 10^4 and V values as μmoles PC released/min./μg. creatine kinase.

ATP^{4-} concentration (σ')	$K_{\sigma'}$	$V_{\sigma'}$
1.0	0.39	0.187
0.66	0.50	0.192
0.5	0.55	0.169
0.33	0.68	0.173

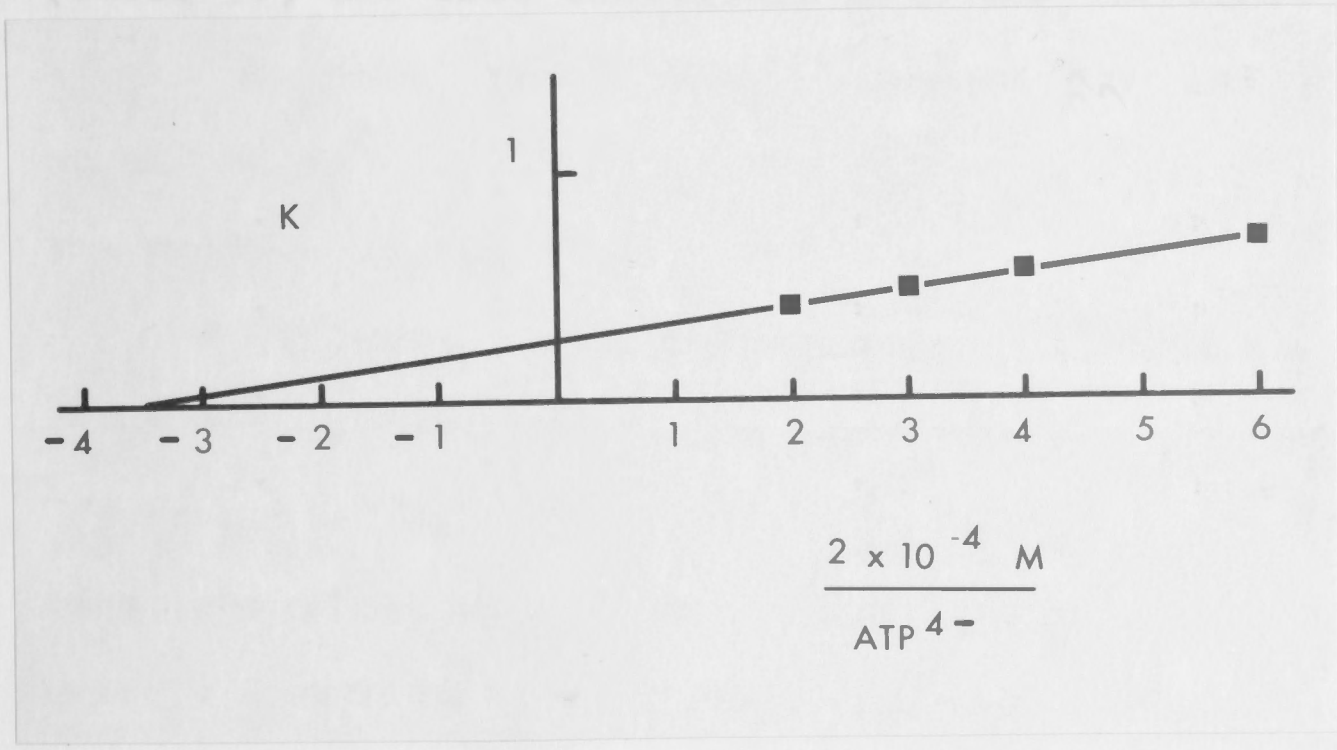


Fig. 43. Plot of the K_σ values from Fig. 42, against the reciprocals of the concentrations of ATP^{4-}.

drawn in Chapter IV for the reverse reaction. Thus, they are consistent with all of the pathways considered being operative in the formation of an active EMS complex. They also indicate that the presence of either Mg^{2+} or ATP^{4-} on the enzyme had little effect on the binding of the other. Those results obtained at pH 8.55 in Section I (Table 33) and at pH 8.0 in Section II (Table 36) are also consistent with the operation of all of the pathways, though they indicated that the presence of one of Mg^{2+} or ATP^{4-} on the enzyme slightly assisted the binding of the other.

A different interpretation emerges from the results at pH 8.4 in Section II when those results are plotted as $^{1}/v$ against $^{1}/Mg^{2+}$ (Fig. 41). In this case the results were consistent with $K_3 = \infty$ (and thus $K_4 = 0$), i.e., no interaction between Mg^{2+} and the enzyme. This would be in agreement with the findings from the inhibition experiments (Chapter V) and is similar to the plots of $^{1}/v$ against $^{1}/Mg^{2+}$ at pH 8.0 reported in Chapter VI (Fig. 31). Again, it was possible to plot the data in Fig. 41 as $^{1}/v$ against $^{1}/ATP^{4-}$ for fixed concentrations of Mg^{2+} and in this way obtain values for K_3 and K_4 (Table 36). If these latter values are of the right magnitude ($K_3 \gg K_4$), they would indicate that the presence of Mg^{2+} on the enzyme assists the binding of ATP^{4-}

($K_4 < K_1$) and vice versa ($K_2 < K_3$). No explanation can be advanced for the finding that different estimates of K_3 and K_4 are obtained according to the manner in which the data is plotted.

For the results reported in Section I, both K_6 and K_1 increased with increasing pH (Table 33), i.e., both $MgATP^{2-}$ and ATP^{4-} were bound less strongly at the higher pH. By contrast, the results in Section II indicated that both K_6 and K_1 decreased (Table 36), so that $MgATP^{2-}$ and ATP^{4-} were bound more strongly, when the pH was increased.

It should be pointed out that these kinetic studies of the forward reaction are far from complete. Only the one experiment has been carried out at pH 8.4 (Section II) and before any definite conclusions can be reached further experiments must be carried out and, if possible, the useful range of free nucleotide and free metal concentrations extended. Moreover, no detailed studies have been made on the effect of creatine concentration on the values obtained for the dissociation constants. If the more recent experimental results can be accepted, the effect of pH is profound (cf. Fig. 40 at pH 8.0 with Fig. 42 at pH 8.4) and it is obviously desirable to study the reaction at a number of pH values.

The agreement between the dissociation constants

reported in this chapter and the thermodynamic values reported by Kuby, Mahowald and Noltmann (1962) is not as good as was observed for the reverse reaction. Thus, for the interaction between ATP^{4-} and the enzyme, the kinetically determined values (K_1) varied between 0.5 and 1.5×10^{-4} M, while Kuby *et al*. (1962) obtained values between 3 and 5×10^{-4} M. For the interaction between $MgATP^{2-}$ and creatine kinase, the agreement was much better; the kinetically determined constant (K_6) varied between 1 and 3.3×10^{-4} M, while Kuby *et al*. (1962) reported a value of 1 to 3×10^{-4} M. The kinetic values consistently indicated that ATP^{4-} was bound more strongly to the enzyme than $MgATP^{2-}$ ($K_1 < K_6$) which is contrary to the findings of Kuby *et al*. (1962).

The results reported in this chapter may also be compared with those obtained by Noda, Nihei and Morales (1960). On the assumption that $MgATP^{2-}$ was the true substrate for the forward reaction and selecting the value of 90,000 M^{-1} as giving the "best fit" for the stability constant of $MgATP^{2-}$, these workers obtained a K_m of 4×10^{-4} M for this complex. They also found that excess ATP inhibited the reaction, which led them to the conclusion that ATP^{4-} was a weak inhibitor of the reaction, though a value for the K_i of ATP^{4-} was not reported. A value of approximately 1 mM for the K_i of ATP^{4-} can be

derived from the plots shown (Fig. 3 of Noda *et al.*, 1960), this being considerably higher than the estimates of K_1 reported above.

SUMMARY

Kinetic studies of the forward reaction catalysed by creatine kinase have been carried out. In general, these have been consistent with the participation of an EM complex in the formation of EMS, though the results of one experiment can be interpreted as indicating that Mg^{2+} did not react at an active site of the enzyme.

CHAPTER VIII

CONCLUSION

CHAPTER VIII

CONCLUSION

The purpose of the investigation, the results of which have been reported in this thesis, was to apply a kinetic approach to the elucidation of the role played by the metal ion, Mg^{2+}, in the reaction,

ATP + creatine $\rightleftharpoons$ ADP + phosphorylcreatine,

catalysed by creatine kinase. The kinetic approach differed from those used previously, in that the concentrations of free Mg^{2+} and free nucleotide were varied by varying the concentrations of total Mg^{2+} and total nucleotide, this necessitating the determination of the stability constants of the complexes, $MgADP^{-}$ and $MgATP^{2-}$, under experimental conditions similar to those used for the kinetic studies.

It was postulated that, because creatine kinase shows an absolute requirement for a divalent metal ion, the reaction proceeds via an enzyme-metal-substrate (EMS) complex. The three possible pathways, which can give rise to this ternary complex, were taken into account and it was considered that the same complex was formed by all pathways. It was also assumed that the reactions leading to the formation of EMS are in rapid equilibrium and that the reaction mechanism is random with respect to the guanidine and nucleotide substrates. The two

latter assumptions appear to be validated by the experimental results.

In summary, it can be stated that the results obtained are consistent with the interaction of the enzyme with $MgADP^-$ and $MgATP^{2-}$ and with the free nucleotides, ADP^{3-} and ATP^{4-}. The evidence for the interaction of Mg^{2+} with the active site of the enzyme was less conclusive, as the results using the original kinetic approach were not always in agreement with those from inhibition studies. Thus, in appraising the results reported in this thesis, particular attention is directed to information concerning the function of Mg^{2+} in the enzymic reaction. The opportunity is also taken to review some recent studies on creatine kinase from other laboratories. After the experimental work had been carried out but before the completion of the writing up of this thesis, the comprehensive review on creatine kinase by Kuby and Noltmann (1962) appeared and reference shall be made to this work during this chapter.

<u>A resumé of the kinetic results reported in this thesis</u>.

All the kinetic studies of the reverse reaction, including those in which either ADP or Mg^{2+} was greatly in excess, were consistent with $MgADP^-$ interacting at the active site and there seems little doubt that this complex can act as a substrate of creatine kinase. The inhibition

experiments clearly indicated that ADP^{3-} competed with $MgADP^{-}$ for the same site. Thus, it is possible that EMS could be formed either by direct interaction between $MgADP^{-}$ and the enzyme or by the enzyme first binding the free nucleotide and then the metal ion.

Most of the studies, using the original kinetic approach, reported in Chapter IV (and in Morrison, O'Sullivan and Ogston (1961)), were obtained using the value of 1,000 M^{-1} for the stability constant of $MgADP^{-}$. The results were consistent with the formation of EMS by all three pathways considered and indicated that the presence of the metal ion on the enzyme did not influence the binding of ADP^{3-} or vice versa. Preliminary experiments, using the value of 4,150 M^{-1} for the stability constant of $MgADP^{-}$ (Chapter II), were also reported in Chapter IV, and in Chapter VI a more detailed study was carried out using this higher value for the stability constant and taking into account the effect of PC on its apparent magnitude. As the higher value is almost certainly the more correct, at least under the experimental conditions, it is felt that more weight should be placed on the results reported in Chapter VI. The importance of the magnitude of the stability constant of $MgADP^{-}$ in its effect on the values obtained for the dissociation constants, and hence on the

interpretation of the results, has been stressed in this thesis and is also mentioned by Kuby and Noltmann (1962). It is clear that the measurement of the magnitude of the stability constant should be made under conditions approximating as closely as possible to those under which the enzymic experiments are carried out, since small errors can cause large variations in the apparent values of K_3 and K_4 and have a smaller, but still significant, effect on K_6 (Chapter VI).

It was not possible, from the results in Chapter VI, to arrive at any firm conclusion concerning the interaction of Mg^{2+} with the enzyme. Some of the results were consistent with no such interaction taking place, as reciprocal plots of velocity against free Mg^{2+} concentration for different fixed values of ADP^{3-} concentration gave the same value for the maximum velocity. However, it was possible to treat this same data by plotting the reciprocal of the velocity against the reciprocal of ADP^{3-} concentration and obtain a real value for the dissociation constant (K_3) of the EM complex. (The plots were defined in each case using maximum velocity and K_m values calculated by the statistical method of Wilkinson (1961)). The actual value obtained is dependent upon a tertiary plot which

gives a direct estimate of the constant, K_4, for the dissociation of EMS into EM and S, allowing K_3 to be calculated. The values obtained for K_4 were small and relatively small differences in this tertiary plot would introduce large errors in the estimates of K_3 (see Discussion, Chapter VI). It is interesting to note that Kuby and Noltmann (1962) recalculated the results reported in Morrison et al. (1961) using the higher stability constant and obtained a value of approximately 5 mM for K_3, which is not markedly different from that of approximately 1 mM reported in both Chapter IV and Chapter VI. Kuby and Noltmann (1962) also recalculated the earlier results of Kuby et al. (1954b) and were able to extract a value of 5 mM for K_3 from the kinetic data.

The inhibition experiments in Chapter V appear to indicate that Mg^{2+} does not react at an active site of the enzyme, as Mg^{2+} was non-competitive with respect to $MgADP^-$. If the contribution to this inhibition by such factors as Cl^- and ionic strength can be considered to be negligible and if the concentration of Mg_2ADP^+ was insignificant, then these appear to be the more convincing results. It was not possible to reconcile these results with the general scheme if EM was considered to participate in the formation of EMS.

The effect of PC on the reaction was also studied in Chapters V and VI. It was found that the PC concentration had no apparent effect on the K_m value for $MgADP^-$ and little effect on the other dissociation constants. This would validate the original assumption that the concentration of PC altered the velocity by a constant factor. It is also indicative of the reaction being random, with respect to PC and nucleotide.

The inhibition by excess Mg^{2+} with respect to PC was not simple and it was possible to obtain different K_i values for Mg^{2+} depending upon whether the enzyme was saturated with PC or the PC concentration was zero. This could indicate that the Mg^{2+} was acting at two independent sites on the enzyme, one independent to the active site and the other such that it affected the K_m for PC (but not the K_m for $MgADP^-$).

A feature of the inhibition experiments was the good correlation between the kinetically determined constants and the equilibrium values reported by Kuby *et al.* (1962) (Table 25, Chapter V). While agreement between the K_m for a particular substrate and its thermodynamic constant cannot be regarded as proof that equilibrium kinetics prevail, the good agreement between the constants for all of $MgADP^-$, ADP^{3-} and PC (and also Mg^{2+}) provides strong evidence in favour of the idea that the reaction obeys equilibrium kinetics,

thus justifying the original assumption.

On the basis of a random reaction obeying equilibrium kinetics, one result from Chapter V cannot be simply explained. The inhibition by excess ADP^{3-} with respect to PC, which was expected to be simple non-competitive, assuming the nucleotide and guanidine moieties to occupy different sites on the enzyme (Kuby et al., 1954b), was found to be slope-linear intercept-hyperbolic non-competitive. It has been pointed out by Dr. W.W. Cleland (personal communication) that the type of hyperbolic plot obtained when the reciprocal of the intercept is plotted against ADP^{3-} concentration (Fig. 29b) is a feature of alternate reaction pathways in steady state mechanisms, where the inhibitor diverts the entire reaction into a different path with a slower rate. Thus, it is possible that pathway III ($M + S \longrightarrow MS \longrightarrow EMS$) is the fastest, but in the presence of excess ADP^{3-} the reaction is forced into pathway I ($E + S \longrightarrow ES \longrightarrow ESM$) which is still operative in the enzymic reaction, though somewhat slower than pathway III. The complex ESM could have a different structure, and thus break down at a different rate, than EMS, or its rate of formation could be different. The mechanism could still be random, but not rapid equilibrium random. Such a mechanism would not be expected to yield linear

reciprocal plots, though the deviation from linearity might not be significant.

The study of the forward reaction catalysed by creatine kinase (Chapter VII) must be regarded as far from complete. The studies at pH 8.0 were consistent with all pathways being operative in the formation of EMS but one experiment at pH 8.4 indicated that there was little, if any, interaction between the metal ion and the enzyme.

Results of recent work on creatine kinase from other laboratories.

The kinetic results of Noda, Nihei and Morales (1960) and Nihei, Noda and Morales (1961) on the forward and reverse reactions, respectively, catalysed by creatine kinase, have been discussed previously. These workers did not consider the possible interaction of Mg^{2+} with the enzyme, but otherwise their findings were in agreement with those reported in this thesis. Thus, the kinetic results were consistent with $MgATP^{2-}$ and $MgADP^{-}$ being the substrates of the reaction. For the reverse reaction, it was necessary to consider ADP^{3-} to be a competitive inhibitor with respect to $MgADP^{-}$, though the effect of ATP^{4-} on the forward reaction was found to be considerably less.

Mahowald, Noltmann and Kuby (1962) studied the

kinetics of the inhibition of creatine kinase by the compounds, 2:4-dinitrofluorobenzene and iodoacetate, which apparently formed stable covalent intermediates at the active site, presumably linked to -SH groups. It was found that the nucleotide substrates, and to a somewhat greater extent, their magnesium complexes, markedly retarded the inhibition, whereas Mg^{2+} alone was ineffective. This indicated that Mg-nucleotide complex and free nucleotide competed for the same site, but could not be regarded as definite evidence against the interaction of Mg^{2+} at the active site, as Mg^{2+} would not be expected to react with -SH groups.

The question of the interaction of a metal ion with creatine kinase has also been investigated by Cohn and Leigh (1962) using magnetic resonance techniques with Mn^{2+} as the activating ion. The results indicated that a ternary complex between Mn^{2+}, ADP and creatine kinase was formed. The parameter measured was the proton relaxation rate of the water in the hydration sphere of Mn^{2+}, the greatest change in this quantity occurring when both ADP and enzyme were added to a solution of $MnCl_2$. A small change occurred when enzyme only was added to the solution of $MnCl_2$, this change being smaller than that observed with serum albumin, while muscle phosphopyruvate hydratase caused a large change

in the proton relaxation rate. These results were interpreted as indicating that the metal ion acted at the active site of phosphopyruvate hydratase, confirming the earlier conclusions of Malmström and his colleagues (Malmström, Vänngard and Larsson, 1958; Malmström, 1953, 1954), but not at the active site of creatine kinase. Further studies showed that the electron spin relaxation rate of $MnADP^-$ was unchanged by the addition of creatine kinase, indicating that the complex did not undergo any deformation when it combined with the enzyme, so that Mn^{2+} was bound only to ADP. As an end result only was observed it would have been possible for a total rearrangement of the ternary complex to have taken place with the same net relaxation rate as was observed with $MnADP^-$ alone. A complicating feature of any studies with Mn^{2+} is that it can form a number of complexes with ADP (Chapter II).

If the activating metal ion does not interact at the active site on creatine kinase, then this would indicate that the metal specificity of this and similar enzymes is largely determined by the configuration of the metal-nucleotide complex. Differences in configuration in the metal complexes have been suggested by Williams (1959) as a possible explanation of the fact that phosphopyruvate hydratase is activated by Mg^{2+} but

inhibited by Ca^{2+} (though it appears certain that the metal does interact at the active site on this enzyme). A comparative kinetic and thermodynamic study of arginine kinase and creatine kinase might provide useful information on metal specificity, as the arginine enzyme is activated by Mg^{2+}, Mn^{2+} and Co^{2+}, while the creatine enzyme is activated by these three metals and also by Ca^{2+}.

Indirect evidence for the reaction of Mg^{2+} at an active site of creatine kinase has come from immunological studies (Samuels, 1961). It was found that both substrates, as well as Mg^{2+}, were required to protect the enzyme against reaction with its antibody; any two of ATP, Mg^{2+} or creatine were ineffective. This led to the suggestion that the "working" enzyme underwent a configurational change which prevented combination with antibody. Samuels, Nihei and Noda (1961) interpreted changes in optical rotation when the enzyme was in equilibrium with all of its substrates as providing evidence for such a configurational change. However, Kuby and Noltmann (1962) considered that these changes were too small for the interpretation to be unquestioned.

<u>A summing up</u>.

It appears that the reaction catalysed by creatine

kinase does not proceed via a compulsory reaction between the free metal ion and the enzyme. In fact, the bulk of the evidence suggests that it is doubtful if an enzyme-metal complex participates in the enzymic reaction at all. As the Mg-nucleotides appear to be bound more strongly than the free nucleotides, the primary function of Mg^{2+} might be to modify the substrate both electronically and structurally so that it is more easily bound to the enzyme.

In the light of this conclusion, it should be possible to assess the kinetic approach which has been developed in this thesis. It was seen that the general approach (Chapter IV) was consistent with all pathways, including that involving EM, being operative in the formation of EMS. Some of the experiments carried out in the reassessment in Chapter VI, could be taken as indicating that the formation of EM was not an activating step, but it was not possible to reach a firm conclusion. However, the evidence from the inhibition studies appeared to be more convincing, as these clearly indicated that Mg^{2+} did not compete with $MgADP^{-}$ for an active site on the enzyme. It is suggested that such a "double-barreled" approach, viz., the use of the general kinetic scheme complemented by the inhibition studies, would be generally applicable to the study of enzymic trans-

phosphorylation reactions.

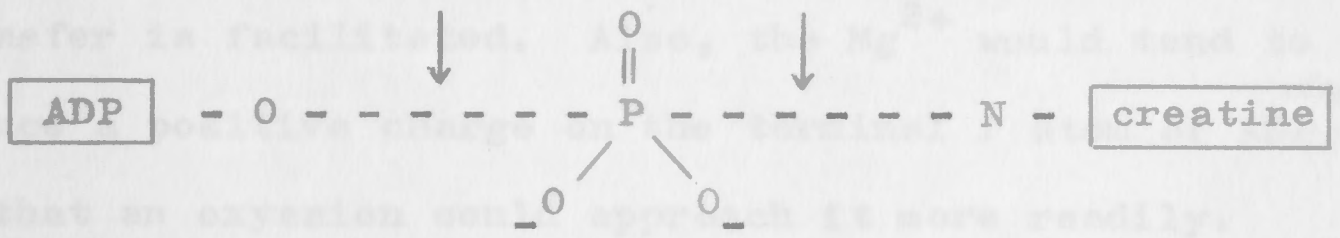
Dr. Mildred Cohn (personal communication) has found evidence from magnetic resonance techniques that for a number of enzymes concerned with the transfer of a phosphoryl group, the activating step is the combination of the metal with the enzyme. The application of the kinetic approach to the study of such enzymes would provide a good test of its general applicability and could be of particular importance for the activation by such ions as Mg^{2+} and Ca^{2+}, which are not easily studied by magnetic resonance techniques.

Some speculations on the transition state of the enzymic reaction.

Most of the kinetic and equilibrium binding data has been consistent with the reaction catalysed by creatine kinase obeying equilibrium kinetics with a random sequence of reactions with respect to nucleotide and guanidine, indicating that the transition state involved the enzyme in combination with all its substrates. This conclusion is similar to that reached by Reynard, Hass, Jacobsen and Boyer (1961) from their studies with pyruvate kinase. These workers proposed a model (Fig. 7 of Reynard et al., 1961) for the transition state of this enzyme in which the terminal phosphate group of ATP occupied the same space as the phosphoryl

group of phosphopyruvate. The reaction was visualised as occurring via the alternate breaking or forming of the P-O bonds of the "common" phosphoryl group.

A similar model could be proposed for creatine kinase, viz.,



(cf. Morrison, Ennor and Griffiths, 1958), with the modification that as an N-P bond is concerned, some rearrangement of the oxygen atoms on the transferable phosphoryl group takes place during the reaction. Alternatively, it could be postulated that the phosphoryl group could rotate around its position on the enzyme or that the creatine site is aligned at approximately right angles to the ADP site.

On the basis that Mg^{2+} is not bound to the enzyme and that the structure of $MgADP^-$ and $MgATP^{2-}$ are the same on the enzyme as in solution, the Mg-nucleotide complex must undergo a rearrangement during the catalytic reaction, as, according to Cohn and Hughes (1962), Mg^{2+} is bound to the α- and β-oxyanions of ADP and the β- and γ-oxyanions of ATP. It would appear that in the forward reaction, the Mg^{2+} weakens the O-P bond by its electron attracting nature, allowing the phosphoryl group to

transfer to the creatine moiety and the Mg^{2+} to "fall back" to the α-group. For the reverse reaction the function of Mg^{2+} might be to attract the oxyanions of the phosphoryl group of PC, bringing it into juxta-position to the terminal phosphorus atom of ADP so that transfer is facilitated. Also, the Mg^{2+} would tend to induce a positive charge on the terminal P atom of ADP so that an oxyanion could approach it more readily. The Mg^{2+} would remain permanently bound to the β-phosphate group and would alternate between the α- and γ-groups according to the direction of the reaction.

APPENDIX

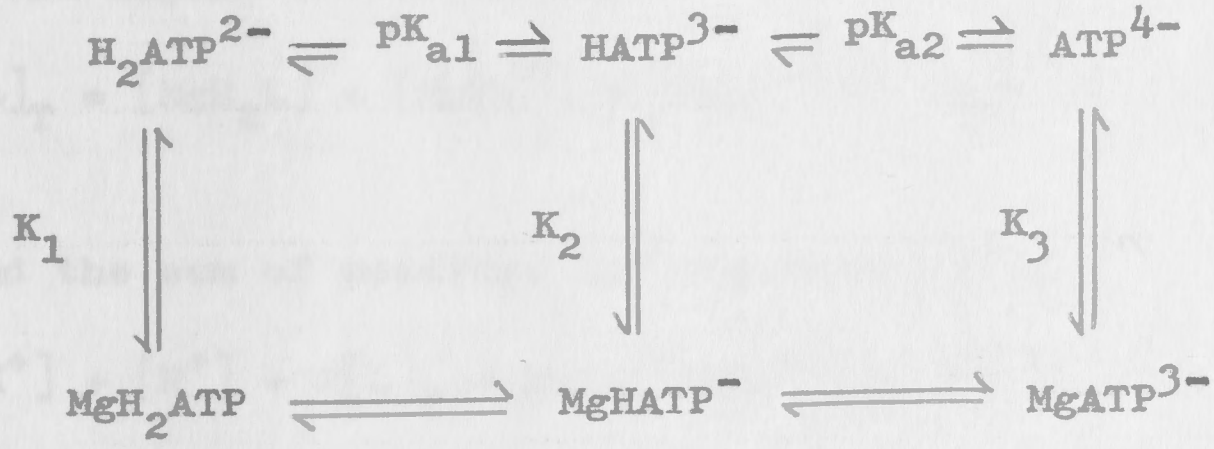
THE CALCULATION OF STABILITY CONSTANTS FOR MAGNESIUM-ATP COMPLEXES USING A 1620 IBM COMPUTER

APPENDIX

THE CALCULATION OF STABILITY CONSTANTS FOR MAGNESIUM-ATP COMPLEXES USING A 1620 IBM COMPUTER

THEORY

Consider the same equilibria as in Chapter II:



K_1, K_2 and K_3 being the stability constants of the appropriate magnesium complexes, defined by

$$K_1 = \frac{[MgH_2L]}{m[H_2L^{2-}]}$$

$$K_2 = \frac{[MgHL^-]}{m[HL^{3-}]}$$

$$K_3 = \frac{[MgL^{2-}]}{m[L^{4-}]}$$

where L = ATP; m = $[Mg^{2+}]$.

The following symbols are used:

$$p = [ATP^{4-}]$$

$$h = [H^+]$$

$$k_2 = \frac{K_2}{K_{a_2}}$$

$$k_1 = \frac{K_1}{K_{a_1} K_{a_2}}$$

Considering total metal concentration,

$$[M]_T = m + [MgH_2L] + [MgHL^-] + [MgL^{2-}] \qquad (i)$$

total ligand concentration

$$[L]_T = [MgH_2L] + [MgHL^-] + [MgL^{2-}] + [H_2L^{2-}] + [HL^{3-}] + [L^{4-}] \qquad (ii)$$

and the sum of positive and negative charges,

$$[K^+] + [H^+] + 2[L]_T + 2m = [MgHL^-] + 2[MgL^{2-}] + 2[H_2L^{2-}]$$

$$+ 3[HL^{3-}] + 4p + [OH^-] + 2[Mg]_T \qquad (iii)$$

(cf. Chapter II)

The following substitutions may be made:

$$[H_2L^{2-}] = \frac{h^2 p}{K_{a_1} K_{a_2}}$$

$$[HL^{3-}] = \frac{hp}{K_{a_2}}$$

$$[MgH_2L] = \frac{K_1 h^2 pm}{K_{a_1} K_{a_2}}$$

$$[MgHL^-] = \frac{K_2 hpm}{K_{a_2}}$$

$$[MgL^{2-}] = K_3pm$$

$$a = 4 + \frac{3h}{K_{a_2}} + \frac{2h^2}{K_{a_1}K_{a_2}}$$

$$b = [K^+] + h + 2[L]_T - [OH^-] - 2[M]_T$$

$$c = 1 + \frac{h}{K_{a_2}} + \frac{h^2}{K_{a_1}K_{a_2}}$$

Then, substituting in equations (i), (ii) and (iii);

$$[M]_T = m + pm\,(k_1h^2 + k_2h + k_3) \qquad (1)$$

$$[L]_T = pc + pm\,(k_1h^2 + k_2h + k_3) \qquad (2)$$

$$b + 2m = k_2hpm + 2k_3pm + ap \qquad (3)$$

From equations (1) and (2),

$$[L]_T - [M]_T = pc - m = d$$

$$\text{i.e. } m = pc - d$$

Substituting for m in equation (2)

$$[L]_T = pc + p(cp - d)(k_1h^2 + k_2h + k_3) \qquad (4)$$

and in equation (3)

$$b + 2(pc - d) = (k_2h + 2k_3)p(pc - d) + ap \qquad (5)$$

so that equations (4) and (5) contain three unknown constants, k_1, k_2 and k_3, and one variable, p.

Expanding equation (4),

$$cp^2(k_1h^2 + k_2h + k_3) + p(c - dk_1h^2 - dk_2h - dk_3) - [L]_T = 0 \quad (6)$$

and equation (5),

$$cp^2(k_2h + 2k_3) + p(a - 2c - dk_2h - 2dk_3) - (b - 2d) = 0 \quad (7)$$

For $b - 2d \neq 0$,

$$(b - 2d)cp^2(k_1h^2 + k_2h + k_3) + p(b - 2d)(c - dk_1h^2 - dk_2h - dk_3)$$
$$= [L]_T cp^2(k_2h + 2k_3) + [L]_T p(a - 2c - dk_2h - 2dk_3)$$

$$cp\left\{(b - 2d)(k_1h^2 + k_2h + k_3) - [L]_T(k_2h + 2k_3)\right\}$$

$$= [L]_T(a - 2c - dk_2h - 2dk_3) - (b - 2d)(c - dk_1h^2 - dk_2h - dk_3)$$

$$p = \frac{[L]_T(a - 2c - dk_2h - 2dk_3) - (b - 2d)(c - dk_1h^2 - dk_2h - dk_3)}{c(b - 2d)(k_1h^2 + k_2h + k_3) - c[L]_T(k_2h + 2k_3)} \quad (8)$$

This value of p may be inserted in equation (7) to give an equation in k_2 and k_3. By selecting points in pairs, two equations in k_2 and k_3 are obtained and hence solutions for k_2 and k_3. Then k_1 may be obtained from equation (6).

PROGRAMME

The programme for the IBM 1620 computer was as follows (programme written by Miss E. A. Reid):

```
C       METAL-ATP COMPLEX FORMATION PROBLEM 73
        DIMENSION TM(2),TL(2),ALK(2),CH(2),H(2)
        DIMENSION A(2),B(2),C(2),D(2)
    2 FORMAT(E15.8,E15.8,E15.8)
    3 FORMAT(I3)
    4 FORMAT(E15.8)
   51 FORMAT(5HERROR,I2)
   52 FORMAT(E15.8,E15.8)
   53 FORMAT(15XE15.8,E15.8,E15.8)
   54 FORMAT(/)
      READ 2, X,Y,Z
      READ 2, CKA1,CKA2
      READ 3, N
      DO 5 K=1,100
      READ 2, CCK2,CCK3
      ACCEPT 4, CCK1
      CK3=CCK3
      DO 6 J=1,N
      DO 7 I=1,2
      READ 2, TM(I),TL(I)
      READ 2, H(I),ALK(I),CH(I)
      A(I)=4.+3.*H(I)/CKA2+2.*H(I)**2/(CKA1*CKA2)
      B(I)=ALK(I)+H(I)+2.*TL(I)-CH(I)-2.*TM(I)
      C(I)=1.+H(I)/CKA2+H(I)**2/(CKA1*CKA2)
      D(I)=TL(I)-TM(I)
      JA=0
      CK1=CCK1/(CKA1*CKA2)
      CK2=CCK2/CKA2
   14 GA=C(I)*(B(I)-2.*D(I))*(CK1*H(I)**2+CK2*H(I)+CK3)
      G=GA-C(I)*TL(I)*(CK2*H(I)+2.*CK3)
      FA=TL(I)*(A(I)-2.*C(I)-D(I)*H(I)*CK2-2.*D(I)*CK3)
      FB=(B(I)-2.*D(I))*(C(I)-D(I)*CK1*H(I)**2-D(I)*H(I)*CK2-D(I)*CK3)
      FL=(FA-FB)/G
      SA=(C(I)*FL**2)*(CK2*H(I)+2.*CK3)-(B(I)-2.*D(I))
      S=SA+FL*(A(I)-2.*C(I)-D(I)*CK2*H(I)-2.*D(I)*CK3)
      IF(JA)8,9,10
    8 L=1
```

```
      GO TO 17
    9 IF(S)11,15,13
   11 L=2
      GO TO 17
   15 L=3
      GO TO 17
   13 JA=1
   16 CK3=CK3+Z
      GO TO 14
   10 IF(S)12,12,16
   12 IF(I-1)21,18,19
   21 L=4
      GO TO 17
   18 ALPHA=CK3+Z
      GO TO 20
   19 GAMMA=CK3+Z
   20 CCK2=CCK2+Y
      CK3=CK3+Z
      JA=-1
      CK2=CCK2/CKA2
   27 GA=C(I)*(B(I)-2.*D(I))*(CK1*H(I)**2+CK2*H(I)+CK3)
      G=GA-C(I)*TL(I)*(CK2*H(I)+2.*CK3)
      FA=TL(I)*(A(I)-2.*C(I)-D(I)*H(I)*CK2-2.*D(I)*CK3)
      FB=(B(I)-2.*D(I))*(C(I)-D(I)*CK1*H(I)**2-D(I)*H(I)*CK2-D(I)*CK3)
      FL=(FA-FB)/G
      SA=(C(I)*FL**2)*(CK2*H(I)+2.*CK3)-(B(I)-2.*D(I))
      S=SA+FL*(A(I)-2.*C(I)-D(I)*CK2*H(I)-2.*D(I)*CK3)
      IF(S)24,25,25
   24 CK3=CK3-Z
      GO TO 27
   25 IF(I-1)22,30,31
   22 L=5
      GO TO 17
   30 BETA=CK3+Z
      FL1=FL
      CK3=CCK3
      CCK2=CCK2-Y
      GO TO 7
```

```
31 DELTA=CK3+Z
 7 CONTINUE
50 Q=ALPHA-GAMMA
   IF(Q)32,33,33
32 Q=-Q
33 R=BETA-DELTA
   IF(R)34,35,35
34 R=-R
35 IF(Q-R)36,36,37
37 CCK2=CCK2+Y
   CK3=BETA+Z
   CCK3=CK3
   ALPHA=BETA
   GAMMA=DELTA
   DO 38 I=1,2
   CK2=CCK2/CKA2
45 GA=C(I)*(B(I)-2.*D(I))*(CK1*H(I)**2+CK2*H(I)+CK3)
   G=GA-C(I)*TL(I)*(CK2*H(I)+2.*CK3)
   FA=TL(I)*(A(I)-2.*C(I)-D(I)*H(I)*CK2-2.*D(I)*CK3)
   FB=(B(I)-2.*D(I))*(C(I)-D(I)*CK1*H(I)**2-D(I)*H(I)*CK2-D(I)*CK3)
   FL=(FA-FB)/G
   SA=(C(I)*FL**2)*(CK2*H(I)+2.*CK3)-(B(I)-2.*D(I))
   S=SA+FL*(A(I)-2.*C(I)-D(I)*CK2*H(I)-2.*D(I)*CK3)
   IF(S)46,43,43
46 CK3=CK3-Z
   GO TO 45
43 IF(I-1)23,48,49
23 L=6
   GO TO 17
48 BETA=CK3+Z
   FL1=FL
   CK3=CCK3
   GO TO 38
49 DELTA=CK3+Z
38 CONTINUE
   GO TO 50
36 FM1=FL1*C(1)-D(1)
```

```
      FM=FL*C(2)-D(2)
C     FL1 AND FM1 ARE THE VALUES OF FM AND FL AT THE 2NTH POINT.
      PRINT 52, FL1,FM1
C     FL AND FM ARE THE VALUES OF FM AND FL AT THE NTH POINT.
      PRINT 52, FL,FM
      PRINT 53, CCK1,CCK2,CK3
      CCK2=2.*CCK2/3.
      CCK3=2.*CCK3/3.
    6 PRINT 54
    6 PRINT 54
    5 CONTINUE
   17 PRINT 51, L
      PAUSE
      END
```

N.B. In the statement 13 lines from the bottom there should be a space between FM1 and ARE so that this should read:

```
C     FL1 AND FM1 ARE THE VALUES OF FM AND FL AT THE 2NTH POINT.
```

Symbols

TM = total [Mg]

TL = total [ATP]

ALK = $[K^+]$

OH = $[OH^-]$

H = $[H^+]$

CKA1 = K_{a_1}

CKA2 = K_{a_2}

CCK2 = K_2

CK2 = $\frac{K_2}{K_{a_2}}$

CCK1 = K_1

CK1 = $\frac{K_1}{K_{a_1} K_{a_2}}$

FL = $[ATP^{4-}]$

FM = $[Mg^{2+}]$

LOGIC

The constants, K_1, K_2, and K_3 are assigned arbitrary values. Pairs of points, designated as the 2nth and nth points, at intervals along the titration curve, were selected for solution.

Using the data at the 2nth point, and with K_1 and K_2 zero, equation (8) is solved for p and the values obtained substituted into equation (7) to give a value, S. If S is positive, K_3 is increased by an increment, z, and the process repeated until S just changes sign. If S is negative, K_3 is decreased by z, again until S changes sign. The value of K_3 obtained to just cause S to change sign is designated α. The process is repeated at the nth point to obtain a second estimate, γ, of K_3.

The whole procedure is repeated using a second value of K_2, this constant being incremented by y. In this way, two further estimates of K_3 are obtained; viz. β and δ at the 2nth and nth points respectively. The estimates of K_3 thus obtained with different values of K_2 are compared:

If $|\beta - \delta| < |\alpha - \gamma|$, the process is repeated, with $K_2 = K_2 + 2y$.

If $|\beta - \delta| \geqslant |\alpha - \gamma|$, the computer prints out the values of K_2 and K_3, and of p and m at the 2nth and nth points.

The entire process is repeated with K_1 increased by an increment, x. In this way a particular pair of points would give values of K_2 and K_3 for each selected value of K_1.

RESULTS AND DISCUSSION

At the start of a computation K_1 and K_2 were set equal to zero, K_3 usually at 50,000 M^{-1}. The increments, y and z, for K_2 and K_3 respectively, were usually set at 100 M^{-1} and x, for K_1, at 10 M^{-1}. It was found that the magnitude of K_1 had very little effect on the value obtained for K_3 but a somewhat larger effect on K_2. In general, sensible results were not obtained with K_1 greater than 20 M^{-1}.

The results reported in Chapter II (Table 5) are shown in Table 38 as printed out by the computer, the free ligand and free metal concentrations being shown as well as the values for the stability constants. The best estimates obtained for K_1, K_2, and K_3 were 20, 500, and 75,000 M^{-1} respectively.

It should be stressed that the programme has not yielded exact results for the stability constants,

TABLE 38

Estimation of the stability constants of Mg-ATP complexes obtained from the IBM 1620 computer

Values are shown as printed out by the computer and have been reported in Chapter II (Table 5).

Typescript from computer				Position of points
+0.00000000E-99RS				
.23107112E-05	.44130915E-03			
.65358832E-06	.69227872E-03			
	.00000000E-99	.40000000E+03	.73500000E+05	20,10
.51964888E-05	.39097898E-03			
.10284096E-05	.48094735E-03			
	.00000000E-99	.56666666E+03	.74200000E+05	25,15
.10788525E-04	.27445823E-03			
.25614249E-05	.39774395E-03			
	.00000000E-99	.47777776E+03	.78166666E+05	31,21
.20866292E-04	.19810303E-03			
.56415696E-05	.35298496E-03			
	.00000000E-99	.41851850E+03	.75777777E+05	36,26
+0.20000000E+02RS				
.23052820E-05	.44027225E-03			
.67390887E-06	.71380219E-03			
	.20000000E+02	.40000000E+03	.74200000E+05	20,10

KEY: $\begin{pmatrix} p \text{ for 2nth point} \\ p \text{ for nth point} \end{pmatrix} \begin{pmatrix} m \text{ for 2nth point} \\ m \text{ for nth point} \end{pmatrix} \begin{pmatrix} \\ K_1 \end{pmatrix} \quad (\quad K_2 \quad) \quad (\quad K_3 \quad)$

but rather indicated the range within which the constants lay. The values reported are the average within this range.

The value for K_3 is in quite good agreement with that of 70,000 M^{-1} obtained by Burton's (1959) method and reported in Chapter II. Both K_2 and K_3 are also similar, though slightly less, than as obtained by the approximation method (cf. Chapter II). The value obtained for K_1 was lower than had been anticipated, though probably subject to fairly considerable experimental error. Finally, it should be noted that the treatment was not extended to consider other species such as $NaATP^{3-}$, Mg_2ATP etc., although it was considered that the formation of such complexes would not greatly affect the estimates obtained.

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